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ANOXIC-ISCHEMIC ENCEPHALOPATHY IN RATS

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The relative susceptibility of gray and white matter to different forms of anoxia is a subject of great interest. While gray matter is generally more vulnerable, there are clinical and experimental observations of anoxic damage to white matter. While pursuing the investigation of this subject, a new technique for producing anoxic cerebral damage in the rat has been developed, and additional data on the sensitivity of white matter to anoxia have been adduced.

Previous workers [1-3] have shown that repeated episodes of histotoxic anoxia (injections of potassium or sodium cyanide) damage the brain with sufficient predilection for white matter to invite comparison with demyelinating diseases (diffuse sclerosis,[1] epizootic leuko-encephalitis of horses,[2] Marchiafava-Bignami disease [3]). Hurst stated: ". . . it is quite evident that as far as the lesions in the white matter are concerned the cyanide does not act *qua* cyanide, but that repeated bouts of anoxia from any cause gradually release destructive forces which, often quite suddenly, involve the white matter in the most serious consequences." [4] Despite the reference to "repeated bouts," Hurst himself reported that an occasional monkey suffered damage to white matter after a single dose of cyanide.[2] Most of the dogs reported by Haymaker, Ginzler and Ferguson [5] suffered severe damage to gray matter with little involvement of white matter following a single dose of cyanide, but one animal had damage exclusively to white matter. Hicks [6] has produced lesions of white matter in rats by a series of intraperitoneal cyanide injections over a period as short as a few hours. Finally, the necessity for postulating gradual release of destructive forces [4] or a "myelinolytic ferment" [3]

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was minimized by the demonstration in this laboratory that a single 30 to 60 minute treatment with cyanide (hydrogen cyanide by respiratory route or potassium cyanide by intravenous route) produced lesions of white matter in rats.[7-9]

In order to explore Hurst's contention that anoxia from any cause could simulate the effect of cyanide, we determined to investigate anoxic anoxia in the rat, following as closely as possible the conditions which proved successful in hydrogen cyanide experiments. However, the rat is not a favorable subject for experiments on anoxic anoxia, and there are few references to its use. Yant and co-workers[10] killed 4 rats in 24 to 72 minutes by displacing oxygen with nitrogen. They reported hyperemia, hemorrhages, edema and damage to certain neurons of the cortex, hippocampus, brain stem and spinal cord. Hicks[6] exposed rats to nitrogen till they were apneic, and after a recovery period the process was repeated 2 to 60 times. Over half the survivors had necrotic lesions in the cortex, gray matter of the corpus striatum, or the substantia nigra. Hicks stated: ". . . there was a narrow margin between a degree of asphyxia sufficient to kill the animal and one that would just fail to produce lesions of the brain."[6]

Our own experiments with nitrogen or nitrous oxide showed that it was difficult to keep a rat in deep anoxia for 20 minutes or more, so as to approximate the conditions of our cyanide experiments. Mortality was high and survivors had no lesions. It seemed likely that the sensitivity of the vital medullary centers was so close to the sensitivity of the remainder of the brain that it was difficult to induce lesions and yet have the total organism survive. Not knowing of any way to diminish the vulnerability of the vital centers, we undertook the reverse—that is, to increase the vulnerability of the forebrain by unilateral common carotid artery ligation. Unilateral carotid ligation by itself did not result in sufficient ischemia to produce lesions.* However, rats prepared in this way and then subjected to anoxic anoxia developed lesions in the homolateral hemisphere. It is the purpose of this communication to describe these anoxic-ischemic lesions and to compare them with cyanide lesions induced in carotid-ligated rats as previously reported.[11]

Methods

Carotid Ligation

Female, 100 to 300 gm., albino Wistar rats were used. Ligation was performed under pentobarbital anesthesia in rats which were exposed to anoxic anoxia on the following day, and under ether in rats which were exposed the same day. The right common carotid was isolated,

* Recently we have had one rat with a small ischemic lesion of the corpus striatum following unilateral ligation alone.

doubly ligated and cut. Some rats showed narrowing of the palpebral aperture due to injury to the sympathetic chain.

Late in the course of these experiments, a blind carotid ligation technique was devised. Under light ether anesthesia a curved needle was inserted into the neck alongside the trachea, till it reached the vertebral column. The point was advanced laterally to the edge of the vertebra and then vertically downward for 1 or 2 mm. The needle was turned laterally and upward to emerge on the lateral aspect of the neck, and the suture was tied externally. Mortality was negligible after initial attempts, during which a few rats died from tracheal ligation, introduction of the needle point into the spinal canal, or laceration of the carotid artery. Narrowing of the palpebral aperture (Horner's syndrome) after arousal from the anesthetic was an invariable concomitant of a successful ligation. If absent, the blind ligation was repeated immediately. Torticollis, due to trauma to muscles and nerves, was frequent. The advantages of blind ligation were that it was very rapid and the ligature could be removed by cutting the suture externally. If the ligature was removed in two hours, blood flow in the carotid was restored; if allowed to remain several days, it often cut through the artery.

Anoxiant Gas Exposure

Nitrogen or nitrous oxide was used to induce anoxic anoxia by displacing air from stoppered 4-liter glass jars containing single rats. It was not possible to maintain the desired degree of anoxia with continuously flowing mixtures of nitrous oxide and oxygen or nitrogen and air, in fixed or variable proportions. The desired results were obtained by admitting pure nitrous oxide or nitrogen slowly, so as to carry the rat past the stage of excitement and hyperpnea into an unconscious state in 5 to 10 minutes. At this point of optimum effect, the gas was turned off completely, and the condition of the rat remained stable in the static atmosphere for at least several minutes. Increasing anoxia (due to consumption of available oxygen) was signaled by decreased respiratory rate. Small increments of air, or of anoxiant gas, sufficed to maintain a fairly stable state for 10 to 30 or even 45 minutes. Close attention to changes in respiration minimized the occurrence of arousals or apnea. Apnea almost always responded to artificial respiration so mortality was negligible.

The static technique did not require special equipment except the tank of gas and flowmeter with reducing valve (a nitrous oxide tank of the type used on anesthesia machines was fitted with a standard oxygen regulator by means of an inexpensive yoke adapter). It was simple to subdivide the inflow of anoxiant gas by a glass manifold so as to expose 6 rats simultaneously in individual jars. The disadvantage of the

static technique was that carbon dioxide accumulated, the amount depending on the size of the rat and the jar, the duration of exposure, and the frequency of increments of fresh nitrous oxide or air. A small rat (100 gm.) in a sealed 4-liter jar showed no hyperpnea after 40 minutes while a large rat (250 gm.) showed increased depth and rate of respiration which could be prevented by introducing a gauze bag of soda lime into the jar. In many of the experiments reported here soda lime was used, but its omission did not influence the results, probably because short exposure times, increments of fresh gases, and decreased metabolic rate due to anoxia prevented accumulation of carbon dioxide.

Nitrous oxide exposures were easier to control than nitrogen exposures. Nitrogen caused more excitement in the early phase of anoxia, often followed precipitously by apnea. Nitrous oxide, probably because of its anesthetic properties, caused less excitement and permitted easier maintenance of an unconscious state with regular respiration and few episodes of apnea. The anoxiant gases were administered once only, except for one experiment with 2 or 3 exposures. The stage of complete unconsciousness, without any response to tilting or tapping the jar, was maintained for 6 to 40 minutes (rarely longer).

Surviving rats usually regained consciousness within a few minutes of removal from the jars. When severe brain damage had been inflicted, some rats showed hemiparesis which was surprisingly mild and which improved with time; there were asymmetry of the limbs when held in vertical position, unilateral lack of resistance to pushing from the side, and occasionally forced circling. Convulsions were not common. Many of the rats with moderate brain damage showed no abnormalities on inspection and superficial examination.

Histologic Techniques

Survivors were sacrificed with chloroform at intervals up to 4 weeks after exposure. The brains were removed, fixed in acetate-buffered 10 per cent formalin, divided into 2 or 3 mm. coronal slices, and embedded in entirety in paraffin. Sections were stained by hematoxylin and eosin, by Luxol fast blue with periodic acid-Schiff-hematoxylin counterstain, and by cresyl violet. The heart and other organs were examined in some experiments.

Results

This study was based on histologic examination of 115 rat brains with lesions induced by surgical carotid ligation followed by the administration of nitrous oxide (83 rats) or nitrogen (32 rats). There were, in addition, 23 rats that survived the procedure without brain lesions; this was not an index of the efficiency of the method because the anoxic exposures were intentionally of minimal severity in most of these cases.

Nine rats died during exposure. Seven rats died after exposure and were discarded because of postmortem decomposition. The brains of an additional 40 rats were studied after blind carotid ligation and nitrous oxide exposure.

Histologic Features

The lesions showed a complete spectrum of gradations in severity and in extent. The most severe lesions were seen in the brains of 23 rats which died or became moribund within 24 to 48 hours of exposure. These animals showed acute necrosis of most of the right hemisphere, including the cortex, corpus striatum, globus pallidus, amygdala, hippocampus, and much of the septum, thalamus and hypothalamus. In some cases the necrosis extended into the right side of the midbrain, and rarely, the pineal was affected. Rarely, there was involvement of the left hemisphere, but only in structures near the midline: medial neocortex, hippocampus and septum. Lesions of lesser severity spared certain parts of the right hemisphere: the septum, corpus callosum and part of the callosal radiation, the medial part of the neocortex, a medial rim of the corpus striatum (adjacent to ventricle), the hypothalamus and parts of the thalamus.

The other rats had less severe lesions, compatible with prolonged survival. The lesions were very variable in location, extent and severity, but were restricted to the right side of the brain. Therefore, the following descriptions refer to the right side only.

Cerebral Cortex. The medial neocortex (cingular cortex) and ventrally located olfactory areas were usually spared. Necrosis of the pyriform cortex and amygdaloid nuclei was variable in occurrence and in extent. The dorsal and lateral neocortex was the region most often completely destroyed (Figs. 1 and 2). Less extensive lesions showed sparing of some dorsal cortex contiguous with the cingular area, and some lateral cortex above the rhinal fissure; in these lesions the deepest layer of cortex escaped necrosis. Still less severe were laminar or patchy areas with partial or complete necrosis of the internal granular or pyramidal cell layers of the dorsolateral cortex (Figs. 3 and 4). The least severe lesions consisted of necrosis or vacuolation of isolated neurons. Mild lesions were sometimes widely distributed, and severe lesions were sometimes focal, but more often, extent and severity showed a direct relationship.

Necrotic neurons showed extreme shrinkage with widened pericellular spaces, nuclear pyknosis and cytoplasmic eosinophilia, followed by fading. Even the mildest lesions showed microglial proliferation and prominent vessels due to endothelial swelling and proliferation. Vacuolation of neurons, glia and intercellular areas occurred in early or mild lesions and on the periphery of severe lesions. Partly or completely necrotic

areas showed loss of myelinated fibers (Fig. 4). Many phagocytes appeared, especially around blood vessels and at the margins of the lesions; their cytoplasm was stained by the periodic acid-Schiff procedure. There were numerous examples of neuronophagia, occasional hemorrhages, rare neutrophils, and no thrombi. In the severest lesions, the tissue architecture was completely destroyed, leaving fragmented and granular eosinophilic debris, or a loose meshwork of fibers, traversed by persisting capillaries and phagocytes. After 3 to 4 weeks, cystic spaces of encephalomalacia were present in areas of cortical necrosis and collapse, and the subarachnoid meshes were widened (Fig. 2).

Corpus Striatum. This structure was of particular interest because it consists of gray matter and myelinated bundles of white matter in intimate admixture. In the most severe lesions there was complete necrosis of both gray and white matter (Fig. 10) except for a narrow rim of preserved tissue along the medial border. Less severe lesions involved only the lateral or central zones; occasionally there were focal necroses elsewhere. When gray and white matter were damaged together, the area of injury to gray matter often extended beyond the area of injury to the white; the reverse occurred occasionally. In some rats the damage was restricted to gray matter (Fig. 9), while others showed exclusive involvement of white matter (Figs. 7 and 8). Rarely there was selective gray matter damage in one part of the striatum and selective white matter damage in another part. The most extensive lesions usually showed the most severe damage, but extent and severity were not always parallel. For example, some areas of total necrosis were very small, while some cases showed relatively mild damage either to gray or white matter extending over most of the striatum. When the striatum was extensively involved, there was sometimes damage to the globus pallidus.

The histologic alterations in gray matter (Figs. 9 and 10) resembled those described in the cortex. The mildest lesion of the white matter consisted of vacuolation, hypercellularity, and loss of affinity for the myelin stain (Figs. 7 and 8). In more severe lesions, the bundles were pale and swollen. In the center of areas of severe necrosis, phagocytes in the white matter originated mainly from surviving capillaries. In milder lesions and on the margins of severe lesions this source was supplemented by microglial hyperplasia in the demyelinated bundles. The resulting hypercellularity of the bundles was the best indicator of selective white matter lesions in hematoxylin and eosin preparations (Fig. 7).

Callosal Radiation. The corpus callosum was not affected except in a few rats that died within 24 hours with massive necrosis. The callosal radiation was damaged in a few rats, but almost always in conjunction with severe necrosis in contiguous tissues on both sides (cortex, hip-

pocampus and corpus striatum.) Even in these severely damaged brains, the radiation showed less damage than contiguous structures (Figs. 1 and 2), and usually exhibited only edema if there was necrosis only on one side. The histologic lesions resembled those described for white matter in the corpus striatum.

Hippocampus. Minimal damage was manifested by necrosis of isolated pyramidal neurons. More severe lesions showed necrosis of a strip of neurons of this layer, beginning a few cells away from the midline, extending laterally along the dorsal lamina (Fig. 12). In more extensive lesions, necrosis involved the entire pyramidal cell layer (both dorsal and ventral portions, Fig. 13), and in some cases, the neurons of the dentate fascia (Fig. 14). Other layers suffered variable damage, but there was usually preservation of myelinated fibers in the alveus and fimbria, but not in the stratum lacunosum (Figs. 15 and 16). The anterior and dorsal part of the hippocampus was affected more than the part which sweeps laterally, ventrally, and posteriorly. In the most severe lesions the entire structure was destroyed, leaving a collapsed mass of glial tissue, vessels and phagocytes.

Diencephalon. Lesions of the thalamus were frequent and variable in location and size. There were irregular, often multiple, zones of necrosis, involving gray matter and interspersed myelinated fibers. The hypothalamus was affected infrequently. A rim of tissue near the midline, and adjacent to ependyma and pia, was usually spared; this included the habenula. Focal necrosis in the pineal was observed occasionally.

Midbrain. The substantia nigra was often damaged. Other parts were involved only in severely affected rats. The necrosis was focal, or involved most of the right half, from the superior colliculus to the substantia nigra, sparing only the subpial and midline regions.

Factors Influencing Distribution and Severity of Lesions. Within the range of 100 to 300 gm., size did not influence the incidence or severity of lesions. This is in contrast to previous work which has shown that selective lesions of white matter due to hydrogen cyanide were more readily produced in large rats.[7] Small rats showed a greater drop in body temperature than large rats following anesthesia and anoxia, but the increased resistance to anoxia attendant upon this mild hypothermia was not of sufficient magnitude to have a major effect on the results.

The lesions were usually less severe and smaller after exposure to nitrous oxide than after nitrogen. This was attributed to more exact control of the depth of anoxia with nitrous oxide, and deliberate attempts to achieve milder damage. All nitrous oxide exposures were done by the static technique. Nitrogen exposures were carried out either by static or

continuous flow techniques; lesions were less severe after static exposures since these were more exactly controlled. Soda lime in the exposure jars did not influence the results with either gas. Nitrogen exposures were made within a few hours of carotid ligation or on the next day; slightly better control was obtained by exposure on the same day. Nitrous oxide exposures were made from one half to 5 hours after ligation, and, within these limits, the postoperative interval did not influence the results. Very short postoperative intervals were avoided, to permit recovery from the drop in body temperature induced by anesthesia.

The severity of lesions was proportional, in overall view, to the duration of exposure, but there were exceptions. For example, massive hemispheric necrosis was produced after as few as 7 minutes of unconsciousness in one rat while another endured 40 minutes without damage. Similarly, the severity of lesions was proportional to the depth of anoxia, but again there were exceptions. For example, apnea, considered a sign of maximal anoxia, was sometimes tolerated without resulting damage when it was of brief duration. It was apparent that depth and duration were two inextricably intertwined factors which determined the extent of brain damage. Similar observations have been made previously with cyanide.[7, 8] The rate of respiration was the main indicator of the depth of anoxia, but it could not be maintained at a constant predetermined level, even with static exposures. Occasionally, transitory return of consciousness occurred, and failure to induce lesions was sometimes attributable to such arousals during otherwise satisfactory exposures. In general, 10 minutes of unconsciousness with a respiratory rate of 120 per minute or more was usually unproductive of lesions, while a similar degree of anoxia extended to 20 minutes often produced mild damage. When the respiratory rate varied between 60 and 120, 10 minutes of unconsciousness caused slight or moderate damage, 20 minutes caused serious damage, and 40 minutes usually resulted in massive necrosis and early death. When the respiratory rate varied between zero and 60, even 10 minutes of unconsciousness caused severe lesions or massive necrosis. These statements are generalizations, and transitory arousals or episodes of apnea or near-apnea, plus individual variation in resistance to anoxia, in distribution of the right carotid system, and in collateral circulation, influenced the outcome.

The large number of rats and the broad spectrum of lesions made it possible to determine an order of sensitivity of different structures to the anoxic-ischemic combination. For this purpose, the experiments with nitrogen were excluded because there were too many instances of massive hemispheric necrosis and no examples of isolated lesions. In the nitrous oxide series, 10 rats with massive necrosis were excluded,

leaving 73 rats which had had ligation by open surgery, exposure by static technique, and had survived more than 24 hours. The distribution of lesions is indicated in Table I; the entries do not take into account extent and severity of lesions except that, as a general rule, more

TABLE I

INCIDENCE AND DISTRIBUTION OF LESIONS

Structures with lesions	No. of rats with lesions	Distribution of lesions by no. of rats and no. of structures affected				
		13 rats 1 structure	13 rats 2 structures	20 rats 3 structures	18 rats 4 structures	9 rats 5 structures
Neocortex	52	0	9	17	17	9
Pyriform cortex	11	0	0	0	2	9
Corpus striatum	41	3	3	8	18	9
Hippocampus	66	8	12	19	18	9
Thalamus	46	2	2	16	17	9

Lesions in 73 rats which survived 24 hours or more with brain damage following right common carotid surgical ligation and exposure to nitrous oxide. Rats with massive necrosis and early death have been excluded. The rats are divided into categories according to the number of the listed structures that were damaged.

severe lesions tended to involve a larger number of structures. The midbrain was not included because small lesions in the substantia nigra were easily missed without serial sections. The figures indicate that the hippocampus was most vulnerable. It was the most frequently damaged region in the entire series (66 of 73 rats), and in the group with minimal damage; i.e., only one structure involved (8 of 13 rats). The neocortex, striatum and thalamus were close to each other in vulnerability. The pyriform cortex was far less vulnerable as it was involved only in the most severe lesions; the midbrain was in the same class, except for the substantia nigra which was probably more vulnerable. The globus pallidus, hypothalamus, olfactory tubercle and septum were less vulnerable.

As a general rule, white matter was more resistant to the anoxic-ischemic combination than gray matter. Among white matter structures, the callosal radiation and anterior commissure were never affected as isolated structures but only with contiguous gray matter; the fimbria and alveus often escaped even when the remainder of hippocampus was destroyed (Figs. 15 and 16). The cerebral peduncle was rarely and mildly affected, and no lesions were observed in the stria medullaris, stria terminalis or hippocampal commissures. Myelinated fibers passing through completely necrotic areas in the cortex or thalamus were destroyed, but even in massive hemispheric necrosis, white matter appeared better preserved than gray matter. Lesions of the corpus callosum were rare, but this is not considered significant because the callosum is a midline structure and may have suffered little or no loss of blood supply.

The most direct comparison of gray and white matter was in the corpus striatum where the two elements are in intimate admixture (Figs. 5 to 10). Among 41 rats with lesions in the striatum, 22 showed involvement of both gray and white matter, but the area of damage to gray matter often exceeded that to the white. There was exclusive damage to gray matter in 12 rats and to white matter in 7. These data suggested that striatal gray matter was slightly more sensitive to the anoxic-ischemic combination than white matter, but it was most interesting that the same experimental procedure was capable of inducing selective gray matter damage in some animals and selective white matter damage in others (Figs. 7 to 9). The determining factors have not been elucidated.

A number of rat brains were studied after blind carotid ligation followed by nitrous oxide exposure. In 29 of these rats the ligature was left in place until sacrifice, or removed 50 to 100 minutes after the anoxiant gas exposure. Of this group, 23 showed lesions comparable to those observed in the rats with surgical ligation. In another group of 11 rats, the ligature was removed immediately after the anoxiant gas exposure. None of these rats showed brain lesions despite anoxiant gas exposures of apparently adequate severity, indicating that it was desirable to maintain the ischemia for a period of time after the anoxic episode in order to produce anoxic-ischemic lesions.

Discussion

Anoxic anoxia and ischemia, each of insufficient intensity to cause lesions when applied separately, proved to be a useful method for producing unilateral cerebral damage in rats when applied together. The chief disadvantage of the anoxic-ischemic technique was that it involved two different forms of anoxia applied simultaneously. While in anoxic anoxia there is only oxygen deficiency, ischemic anoxia involves deficiencies of oxygen, glucose and other materials and is complicated by accumulation of waste products. Scholz[12] stated that the cerebral and cerebellar cortices were more susceptible than the basal ganglia under ischemic conditions, whereas the reverse order applied under hypoxemic conditions. Despite these differences, the histologic effects of both forms of anoxia could be similar[13] or identical.[14] Direct observation of the living brain by Denny-Brown and Meyer showed that anoxic anoxia produced the same effects as ischemia and that "the vascular damage caused by ischemia is mediated by anoxia."[15] In addition, it is probable that anoxic anoxia is always complicated by superimposed scattered foci of ischemia, thus explaining the tendency for anoxic anoxia to cause focal rather than uniform lesions in a vulnerable system.[12] The anoxic-ischemic experiments described here illustrate Scholz's conten-

tion that the distribution of anoxic lesions depends both on a "vascular factor" and a "systemic factor" (tissue vulnerability, or *"pathoklisis"*);[12] the carotid ligation constituted a "vascular factor" which localized the effects of the subsequent anoxic anoxia to the right cerebral hemisphere.

The combination of ischemia and anoxic anoxia is, therefore, not merely an artifice devised to produce lesions in the rat brain but has its counterpart in human disorders. Neurologic sequelae of surgical anesthesia with nitrous oxide were attributed by Courville to anoxic anoxia,[16] but the occurrence of hemiparesis and focal or asymmetrical cortical lesions implicated a vascular factor. Bourne attributed deaths and sequelae following dental anesthesia with nitrous oxide to fainting attacks which, in the upright position, led to hypotension and cerebral ischemia; he added that "sometimes lack of oxygen in the anaesthetic mixture may have played an important part."[17] Monrad-Krohn attributed cerebral palsy to combined forms of anoxia.[18] Courville attributed general, hemispheric, and lobar atrophies, microgyria, and some instances of porencephaly to the association of ischemia and anoxic anoxia.[14]

The anoxic-ischemic technique caused damage to gray more than white matter in rats. As in other species, selective neuronal necrosis in the hippocampus, laminar necrosis in the cortex and focal necrosis in the basal ganglions and other areas constituted the hallmarks of anoxia. Of greater importance was the demonstration that selective lesions of white matter were induced in a few rats by the combination of anoxic anoxia and ischemia. It is pertinent to compare these results with those of Morrison[19] who produced lesions of white matter in monkeys and dogs by anoxic anoxia. In his experiments, white matter lesions occurred only after repeated anoxic episodes more severe or more numerous than those required for damage of gray matter. In the present experiments (as in previous work with cyanide[7-9, 11]) a single exposure was effective. Lesions of white matter following single episodes of anoxic anoxia have been reported in human subjects also (nitrous oxide,[16, 20] high altitude[21]). Therefore, constant repetition of anoxia is not as essential for the production of demyelinating lesions as Morrison[19] and Hurst[4] have indicated. On the other hand, the present results support Hurst's statement[4] that anoxia from any cause can damage white matter.

The lesions produced in rats by the anoxic-ischemic technique can be compared with those produced by hydrogen cyanide after carotid ligation.[11] The two procedures are comparable in that an attempt was made to maintain a fairly constant sublethal degree of anoxia. The reactions during cyanide exposure differed in that respiration was slower and deeper, the ear twitch after a brisk tap on the jar persisted longer, and

respiratory depression often failed to respond to artificial respiration. The same structures were injured by both techniques except that the corpus callosum was spared in the anoxic-ischemic experiments, probably because of its midline location; it is unlikely that unilateral carotid ligation caused appreciable impairment of its blood flow. The anoxic-ischemic experiments produced fewer selective lesions of white matter than the cyanide-ischemic experiments. This can be explained, at least partly, by the paucity of cytochrome oxidase in white matter which makes it particularly susceptible to cyanide. The lesions resulting from the anoxic-ischemic procedure were almost always unilateral, whereas the cyanide-ischemic procedure yielded bilateral lesions in white matter frequently and in gray matter moderately often. It is possible that the adverse influence of decreased blood flow during cyanide exposures was partly mitigated by the decrease in the amount of cyanide reaching the ischemic hemisphere. Bilateral lesions might result when the discrepancy in cyanide levels in the two hemispheres was just counterbalanced by the effects of ischemia.

Summary

A method has been described for producing unilateral anoxic lesions in the rat brain by combining anoxic anoxia (a single exposure to nitrous oxide or nitrogen) and ischemia (unilateral common carotid ligation). The ligations were performed surgically or by a blind technique. The lesions involved cerebral cortex, corpus striatum, callosal radiation, hippocampus, thalamus and midbrain. The extent and severity of the lesions were very variable; the depth and duration of the anoxic period were the chief determining factors. The hippocampus was the most vulnerable structure, and gray matter was generally more vulnerable than white matter. Lesions in the corpus striatum usually affected both gray and white matter but in some rats the gray matter and in others the white matter were selectively damaged. The selective lesions of white matter, although less frequent than those produced by cyanide, provided additional evidence for the concept that any form of anoxia can produce demyelination. However, the results obtained with the anoxic-ischemic combination (like those obtained previously with cyanide) showed that repetition was not an essential factor for anoxic damage to white matter.

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LEGENDS FOR FIGURES

Figures 1 to 4: Anoxic-ischemic lesions of the cerebral cortex.

FIG. 1. Coronal section of rat brain, showing anoxic-ischemic lesions of 27 days' duration. Cystic encephalomalacia and shrinkage of the right neocortex (and corpus striatum) have resulted in an asymmetrical appearance. Luxol fast blue-periodic acid-Schiff-hematoxylin stain. × 5.

FIG. 2. Higher magnification of Figure 1, showing cystic encephalomalacia involving full thickness of the cortex. The underlying white matter (callosal radiation) is preserved (left side). Luxol fast blue-periodic acid-Schiff-hematoxylin stain. × 125.

FIG. 3. Laminar necrosis of the neocortex. Most of the neurons in the necrotic layer are shrunken and faded. The hypercellularity is due to microglial proliferation. Hematoxylin and eosin stain. × 125.

FIG. 4. Another area of laminar necrosis. A few surviving neurons in the damaged layer are seen. The pallor of this layer is due to destruction of myelinated fibers. Luxol fast blue-periodic acid-Schiff-hematoxylin stain. × 125.

Figures 5 to 10: Anoxic-ischemic lesions of the corpus striatum.

FIG. 5. Normal area showing a bundle of white matter with oligodendroglial nuclei (right side) and gray matter with many neurons. Hematoxylin and eosin stain. × 500.

FIG. 6. Normal area showing a white matter bundle with a normal affinity for myelin stain (right side). Luxol fast blue-periodic acid-Schiff-hematoxylin stain. × 500.

FIG. 7. Selective damage to a white matter bundle which shows hypercellularity due to microglial proliferation (right side). Gray matter appears normal. Hematoxylin and eosin stain. × 500.

FIG. 8. Selective damage to a white matter bundle which shows loss of affinity for myelin stain (right side). Luxol fast blue-periodic acid-Schiff-hematoxylin stain. × 500.

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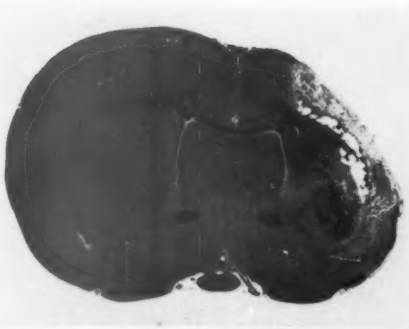

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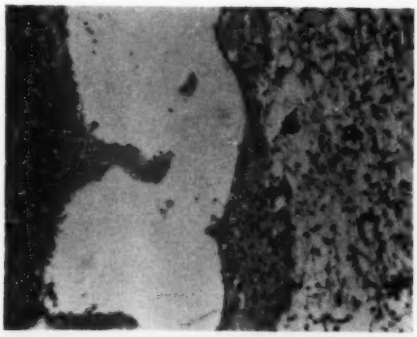

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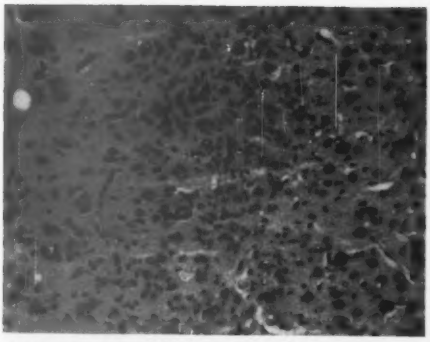

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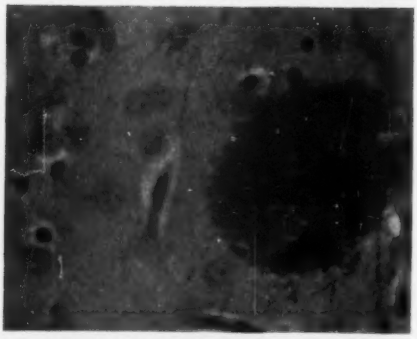

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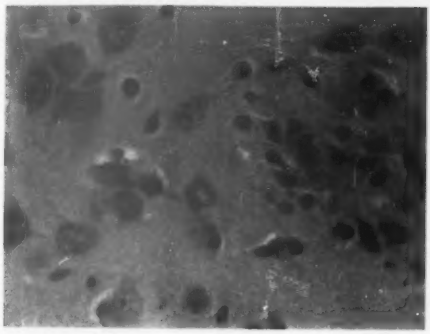

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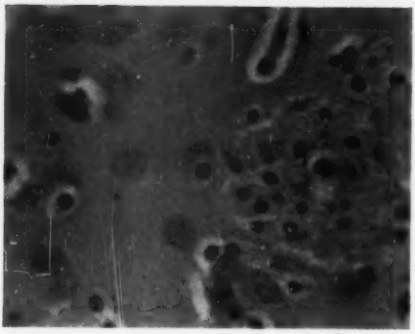


FIG. 9. Selective damage to gray matter. The neurons are extremely shrunken and either pyknotic or faded. There are a few astrocytic nuclei which appear normal. A bundle of white matter appears normal (right upper quadrant). Hematoxylin and eosin stain. × 500.

FIG. 10. Damage to gray and white matter. The neurons are faded and are barely discernible. The white matter bundle shows edema and microglial proliferation (right side). Hematoxylin and eosin stain. × 500.

Figures 11 to 16: Anoxic-ischemic lesions of the hippocampus.

FIG. 11. Normal hippocampus (for comparison). Hematoxylin and eosin stain. × 20.

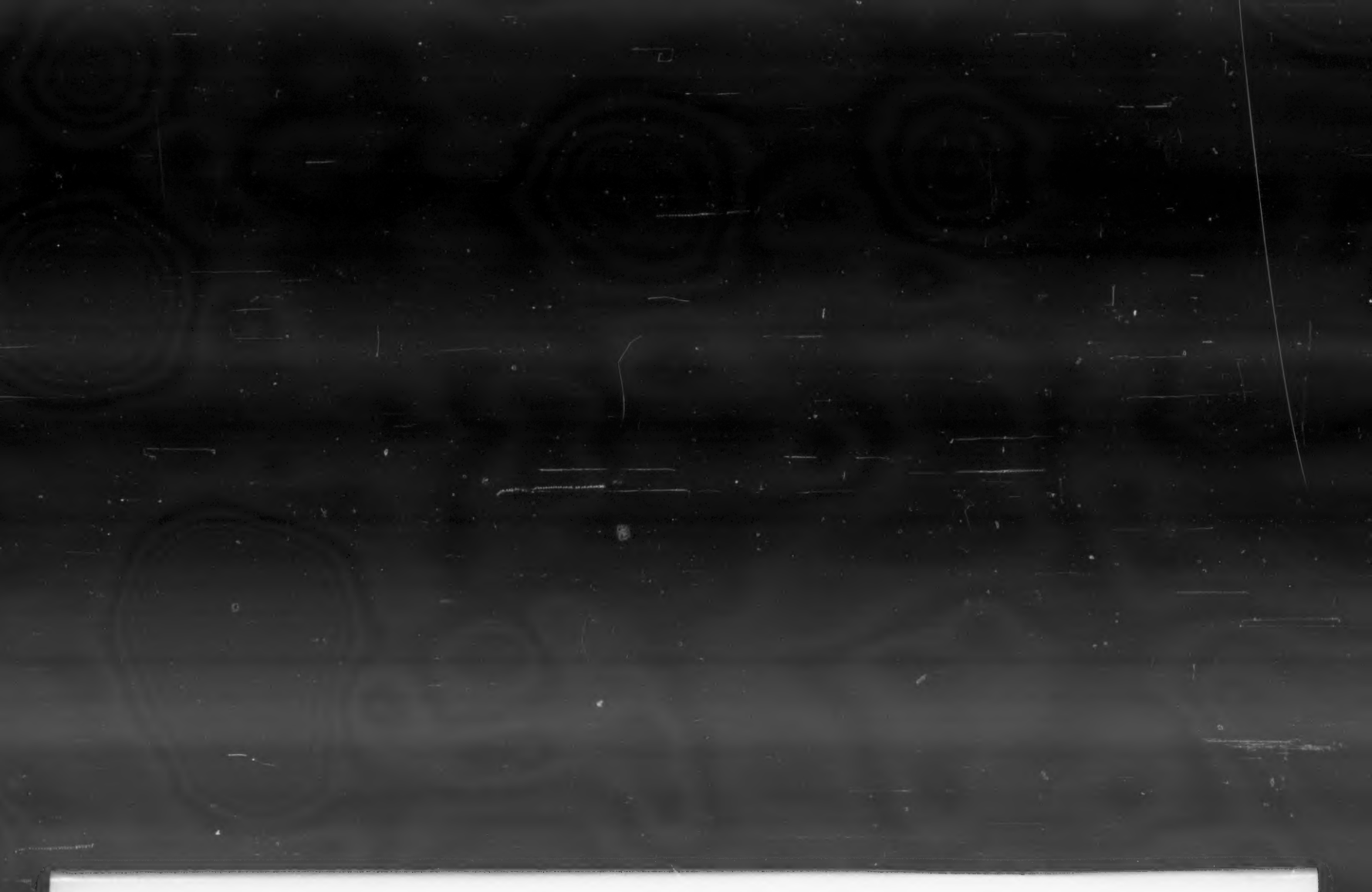
FIG. 2. The dorsal portion of the pyramidal cell layer is necrotic. Hematoxylin and eosin stain. × 20.

FIG. 13. The entire pyramidal cell layer is necrotic, but the dentate layer is preserved. Hematoxylin and eosin stain. × 20.

FIG. 14. Almost the entire hippocampus, including both pyramidal cell and dentate layers, is destroyed. Hematoxylin and eosin stain. × 20.

FIG. 15. Same block as Figure 14. Despite severe destruction of the hippocampus, the alveus and fimbria retain normal affinity for myelin stain. Luxol fast blue-periodic acid-Schiff-hematoxylin stain. × 20.

FIG. 16. Higher magnification of Figure 15 to show destruction of the pyramidal cell layer (below) but preservation of myelinated fibers of the alveus (darkly stained layer just below the horizontal row of ventricular ependymal cells). × 125.



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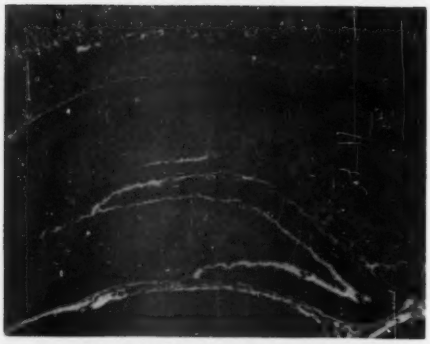

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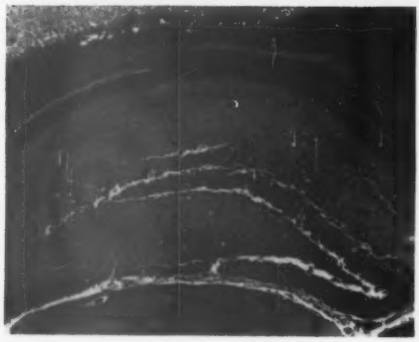

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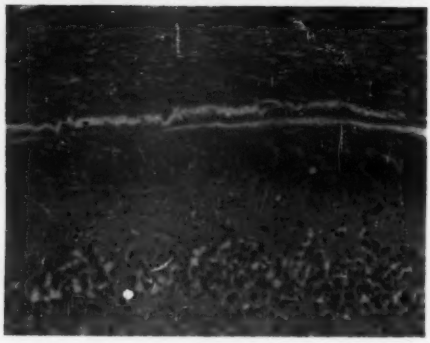

16

AN ELECTRON MICROSCOPIC STUDY OF EXPERIMENTAL ATHEROSCLEROSIS

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Long[1] has reviewed the early history of the efforts to determine the etiology of human atherosclerosis. The early work was characterized by analyses of the morbid histology, for the most part by such 19th century pathologists as Virchow, Rokitansky, Lobstein and Jores. Biochemical and experimental approaches can be dated from 1908, the year when Ignatowsky began feeding experiments on laboratory animals. These experiments were extended by Anitschkow,[2] who succeeded in inducing lesions in rabbits by feeding cholesterol. This work has been further extended by Katz and Stamler,[3] Altschul,[4] Leary,[5] Duff,[6] Gofman and co-workers,[7] and others. A comprehensive discourse on the pathogenesis of experimental atherosclerosis is beyond the scope of this paper. A number of excellent recent reviews can be found in the works of Katz and Stamler,[3] Altschul,[4] Duff,[6] Klotz,[8] Bailey,[9] Dock,[10] and Page.[11]

The purpose of the present paper is to report some additional and very early alterations occurring within the coronary arteries of rabbits fed cholesterol experimentally. Use has been made of the electron microscope, in anticipation that its superior resolving power would contribute to an understanding of the anatomic alterations and perhaps shed some light upon their pathogenesis. An effort has been made to correlate this study with observations made with the light microscope. Because the amount of tissue which could be examined within the time available was limited by technical factors inherent in electron microscopy, the number of animals which could be examined was less than was desirable. For this reason, some of the observations should be interpreted with caution and regarded as tentative and preliminary until confirmed.

An understanding of the submicroscopic structure of the normal coronary arteries is necessary before one can interpret the pathologic changes induced by cholesterol feeding. An investigation of the normal structure of coronary arteries in 6 rabbits has been completed.[12] These animals can be regarded as comprising part of the control series for the present study. Further details of the submicroscopic features of normal

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coronary vessels can be found in the report of Moore and Ruska.[13]

Buck [14] has published an electron microscope study of atherosclerotic changes induced in the aorta of rabbits by feeding cholesterol. Many of his findings are confirmed in the present investigation.

Material and Methods

Rabbits were selected for this investigation with two purposes in mind. First, they develop intimal lesions with lipid feeding.[2, 4, 15-18] Second, these animals have been used by a great number of investigators in the past, and it was felt that correlations with the present study could be accomplished with greater validity if rabbits were chosen.

Little attention was paid to age or body weight of the animals, as it has been shown by Duff [6] and Hueper [19] that these factors have little direct influence on the type and severity of experimental atherosclerosis. Females were used because Fillios and Mann [20] have pointed out that females show greater hypercholesterolemia after cholesterol feeding than do males.

Ten mature female albino rabbits were used, in addition to the 6 normal control animals described previously.[12] The experimental procedures and the principal observations in each rabbit are summarized in Table I.

Six of the 10 rabbits were chosen for examination by electron microscopy. They were placed on a cholesterol diet, as described below. At the time of sacrifice each was anesthetized with ether (U.S.P.). Heart tissue was prepared for electron microscopy and was examined as described earlier.[12]

All 6 of the rabbits in these feeding experiments received alfalfa pellets coated with cholesterol. As suggested by Buck,[21] 70 gm. of cholesterol (melting point, 148° to 150° C.) was dissolved in one pound of ether. The pellets were soaked briefly in the solution. The ether was then allowed to evaporate. The exact quantity of cholesterol fed to each animal was not determined, but this was not deemed to be critical in a study concerned exclusively with morphologic changes.

Three of the 6 rabbits were also given large doses of supplementary lipid in the form of an emulsion of cholesterol in cottonseed oil (1 gm. of cholesterol in 10 cc. of oil) by means of a stomach tube over a period of 24 to 48 hours. These were designated rabbits 1, 2 and 3. Rabbit 4 received this same emulsion in capsules for 15 days. The fifth animal was given intravenously over a period of 48 hours an emulsion of 2.5 per cent cholesterol stabilized in 0.5 per cent sodium stearate. The sixth rabbit was fed coated pellets for 104 days but received no supplementary lipid.

Four additional rabbits were sacrificed for light microscopy, and were assigned the numbers 7 to 10. Rabbit 10 was fed no fat and served as a control. As stated above, 6 additional control animals were examined in another study.[12] Rabbits 7, 8 and 9 were fed alfalfa pellets with cholesterol in a manner similar to the feeding of rabbits 1 to 6. Rabbits 7 and 8 were, in addition, given 10 per cent cholesterol in cottonseed oil by stomach tube 48 hours prior to sacrifice. Rabbit 9 was fed coated pellets for 104 days, but was given no supplementary lipid. At the time of sacrifice, rabbits 7 to 10 were anesthetized with ether, the thoraces and pericardia were opened, and 30 cc. of cold normal saline was injected into the left ventricle of each. The main branches of the right and left coronary arteries were dissected free. A portion of the tissue from each heart was frozen and sectioned without prior fixation. The remainder was first placed in 10 per cent formalin for 4 days and then in Helly's solution for 6 hours; blocks were embedded in paraffin.

The frozen sections were stained immediately with oil blue N, using the supersaturated isopropanol method of Lillie[22, 23] to demonstrate lipids. The tissue embedded in paraffin was sectioned at 5 to 7 μ and stained for elastic tissue with orcinol-new fuchsin, as described by Fullmer and Lillie.[24]

The experimental procedures imposed on each rabbit are summarized in Table I.

Observations

Normal Structure

Figure 3 shows a low-power electron micrograph of a section through the wall of a normal rabbit coronary artery. A more complete description of the normal structure can be found elsewhere.[12] The principal features of the normal rabbit coronary are summarized and prepared in the form of a diagram (Fig. 1). It is presented to provide convenient comparison with pathologic alterations induced by lipid feeding.

Alterations Induced by Cholesterol Administration

The pathologic features observed are summarized in Table I. Specimens from all 9 experimental animals receiving cholesterol showed striking alterations in the endothelial cells and internal elastic lamina. Figure 2 has been prepared to summarize these lesions. The most apparent lesions occurred in discrete areas along the internal elastic lamina. These are called initial elastic lesions (ES). They appeared as early as 24 hours after cholesterol feeding. They were small focal lesions, measuring up to 10.5 μ in diameter, characterized by decrease in density and swelling of the elastic components. A second, but less

TABLE I

SUMMARY OF OBSERVATIONS

Rabbit no.	Cholesterol feeding: Duration of feeding cholesterol-coated pellets	Cholesterol feeding: Supplementary lipid *: Amount	Cholesterol feeding: Supplementary lipid *: Method of feeding	Anatomic alterations observed: Dense endothelial caps	Anatomic alterations observed: Initial elastic lesions	Anatomic alterations observed: Medial alterations	Anatomic alterations observed: Elastic tissue alterations in adventitia	Instrument used
1	24 hours	4.8 gm. cholesterol 42 cc. cottonseed oil	Stomach tube	Present	Present	Not seen	Not seen	Electron microscope
2	48 hours	7 gm. cholesterol 60 cc. cottonseed oil	Stomach tube	Present	Present	Not seen	Minimal	Electron microscope
3	24 hours	7 gm. cholesterol 40 cc. cottonseed oil	Stomach tube	Present	Present	Minimal	Minimal	Electron microscope
4	25 days	20 gm. cholesterol 10 cc. cottonseed oil	Gelatin capsules	Present	Present	Not seen	Moderate	Electron microscope
5	48 hours	2 gm. cholesterol	Intravenous inj.	Present	Present	Minimal	Severe	Electron microscope
6	104 days	None		Present	Present	Marked	Moderate	Electron microscope
7	48 hours	7 gm. cholesterol 60 cc. cottonseed oil	Stomach tube	Fat material near endothelial cells	Elastic stain defects	Not seen	Not seen	Light microscope
8	48 hours	7 gm. cholesterol 60 cc. cottonseed oil	Stomach tube	Fat material near endothelial cells	Elastic stain defects	Not seen	Not seen	Light microscope
9	104 days	None		Fat within plaques	Elastic fibers in plaques	Moderate	Not seen	Light microscope
10	No feeding (control) †	None	Uncoated pellets	None	None	None	None	Light microscope

* Supplementary lipid administered as indicated in addition to the amount of cholesterol fed in the form of coated alfalfa pellets.
† Six additional control rabbits were studied and described in another paper.[12]

frequent finding, is also depicted in Figure 2. This consisted of accumulations of dense material associated with the endothelial cells (EC).

In the present study there seemed to be little correlation between the length of feeding and the severity of these lesions. This is in agreement with the observations of Altschul[4] and Duff.[6] Moreover, Heath[25] and Duff[6] have stated that the rapidity of development of vascular lesions may depend somewhat on the physical state of the cholesterol and the means by which it is given. In general agreement with these observations, rabbit 5, which received cholesterol intravenously, showed more extensive alterations than did animals 1, 2 and 3, which were fed comparable amounts by the gastrointestinal route.

Lesions Associated with Acute Lipemia

Endothelial Cells. Observations were made with the light microscope on frozen sections stained for lipid from rabbits 7 and 8, which were sacrificed during acute lipemia induced by feeding cholesterol by stomach tube. These sections revealed small (up to 7 μ) stained globules in and upon endothelium (Figs. 4 and 5). It was difficult to localize the lipid material with the light microscope, but it appeared to be upon, within, and beneath the endothelial cells, as well as on and near the internal elastic lamina.

With the electron microscope, characteristic dense bodies or caps were found overlying portions of the lumen surface of a few endothelial cells (Figs. 6 to 9, EC). These appeared to have an inhomogeneous, somewhat porous structure. Some of these bodies enclosed irregularly shaped vacuoles (Figs. 8 and 9). In one such structure, numerous dense, irregular particles were found ringing vacuoles (Figs. 8 and 9). It is possible that the vacuoles represented the sites of lipid which was removed by solvents in preparing the tissue.

Similar dense material was found in numerous indentations of the plasma membrane, termed *"caveolae intracellulares,"* * and in vesicles found immediately beneath them (Figs. 6 to 9, X, U). This dense material was also found in occasional branching cavernous structures seen extending deeply into the endothelial cytoplasm (Fig. 6, W). On close examination it could be seen that the cytoplasmic membrane of the endothelial cells, wherever associated with this dense material, was intact and discretely resolvable.

Elastic Tissue. Characteristic alterations were observed with the elec-

* The term *"caveola intracellularis,"* meaning small intracellular pit or cave, was introduced by Yamada.[26] The term is defined as a "small pocket, vesicle, cave or recess communicating with the outside of a cell and extending inward, indenting the cytoplasm and the cell membrane. Such caveolae would be lined with extensions of the cell membrane, but could be pinched off to form free vesicles within the cytoplasm."

tron microscope in the internal elastic lamina of all the rabbits given cholesterol, including those sacrificed during acute lipemia without a prolonged cholesterol diet. So far as could be determined, these changes have not previously been described. They appeared to be very early lesions indeed, since they were found after only brief acute lipemia. Therefore, they have been termed initial elastic lesions.

The initial elastic lesions appeared as discrete focal areas in the internal elastic lamina. They were characterized by a decrease in density and by bulging or swelling of the elastica interna, with loss of its characteristic fibrillar nature (Figs. 14 to 22, ES). Normally, the internal elastic lamina was observed to have numerous submicroscopic fibrils, 500 Å in diameter, embedded within its matrix. These fibrils are below the limit of resolution of the light microscope. The initial elastic lesions were small in size, measuring from 1.8 μ to 10.5 μ across.

The characteristic bulges of the lesions might protrude from either the upper or the lower surfaces of a region of the elastica interna, which otherwise appeared normal, or they might billow from both surfaces of a focal lesion involving the entire thickness of the elastic layer. In some instances, they pushed the endothelial cell cytoplasm toward the lumen (Figs. 14 to 18, 20 and 22) or encroached upon the cytoplasm or even the nuclei of smooth muscle cells (Figs. 16 to 19, and 22). The initial elastic lesions were interspersed with expanses of normal elastic tissue (Figs. 16 and 17, I).

When sections of tissue obtained from rabbits sacrificed during acute lipemia were stained for elastic tissue and viewed with the light microscope, small (4 μ), discrete defects in the staining quality along the internal elastic lamina were observed (Fig. 11, Q). These probably corresponded to the initial elastic lesions seen with the electron microscope. Infrequently, other portions of the elastica interna appeared thicker than normal and stained less intensely (Figs. 12 and 13). It could not be determined if these defects in staining corresponded to the deposits of lipid in the internal elastic lamina (Figs. 4 and 5).

Figures 20 and 21 show a large ovoid defect within the cytoplasm of an endothelial cell. This defect contains material resembling that found in the initial elastic lesions. This appearance is interpreted as representing a tangential section through a bulge of an adjacent initial elastic lesion. Figure 22 illustrates still more severe lesions. Here there is fragmentation of the elastica interna in addition to extensive swelling and loss of density and fibrillar structure.

Similar changes were noted occasionally in the elastic tissue of the adventitia. These were most conspicuous in the animal given cholesterol intravenously (Fig. 23, ES). Such elastic lesions do not seem to be

limited to blood vessels alone. Swellings in the elastic tissue lying under the epicardium were noted in a rabbit fed cholesterol for 18 days.

Media. A third lesion seen, although with less frequency (except in the animal fed cholesterol for 3 months, rabbit 9), was characterized by defects in the arrangement of smooth muscle cells in the media. These areas were devoid of smooth muscle cells but were occupied by collagen fibers (Fig. 24, C). Often the internal elastic lamina above was unchanged. No fibroblasts could be identified in these regions.

In the perinuclear regions of a few smooth muscle cells of one animal (no. 3), there were found large, ovoid vesicles (0.9 μ in diameter) with irregular dense borders and 2 or 3 lamellas running diagonally across them (Fig. 25, arrow).

Alterations in Animals Not Subjected to Acute Lipemia

Two animals (rabbits 6 and 9) were given a high cholesterol diet for 3 months and were sacrificed without the induction of acute lipemia. Coronary vessels from rabbit 9, as prepared in frozen sections stained for fat, showed large plaques, which, in some areas, almost obliterated the coronary lumen (Fig. 26). The plaques, which were similar to others described in the literature,[2, 6, 9, 15, 21, 25, 27] contained large amounts of stainable lipid which was distributed both intracellularly and extracellularly. The exact nature of the cells was not clear. Elastic tissue stains revealed no lesions of the internal elastic lamina (Fig. 27, I). The plaques contained some elastic fibers, as well as large and small vacuoles both within and outside cell boundaries.

Sections for the electron microscope were taken through such plaques in rabbit 6. The cells, most of which resembled smooth muscle cells, were fusiform to ovoid in profile, with elongated nuclei, mitochondria and vesicles in the perinuclear zone, dense ovoid areas elsewhere in the cytoplasm (Fig. 29) and numerous large and small vacuoles (Figs. 28 to 30, V). A basement membrane surrounded each cell, and myofilaments were not clearly defined. Some of the cells, however, held no resemblance to smooth muscle. A few were elongated and exhibited considerable amounts of endoplasmic reticulum (Fig. 30). Others contained large, ovoid cytoplasmic densities in which were numerous irregularly shaped vesicles (Fig. 31, U). Within cells and extracellular spaces, there were vacuoles or rounded defects with densities close to that of the embedding plastic (Figs. 28 to 30, V). These could represent the original sites of lipid which had been removed by solvents in preparing the tissues.

The lumen borders of the plaques were lined by intact endothelium (Figs. 32 to 34, A). Occasionally, dense material, similar to that seen in rabbits with acute lipemia, was observed upon the endothelial cells.

Such material could not be recognized definitely within the plaques. In some areas endothelial cells contained large vacuoles (Figs. 33 and 34, V).

Overlying the plaques, subendothelial elastic components were present only as scanty fragments or were absent (Figs. 32 and 33, E); near the periphery, however, more normal-looking internal elastic fibers could be found (Fig. 34, I). In these regions, near the periphery of the plaques, there was an increase in the intercellular elastic tissue, which was fragmented but not swollen (Fig. 32, E). In some areas a thick sheet of elastic tissue was noted to traverse the base of the plaque at a deep level, separating the plaque from the underlying relatively normal-appearing media (Fig. 33, I). Conventional sections from rabbit 9, stained for elastic tissue, showed the deep running sheet of elastic tissue to be continuous with the normally positioned subendothelial internal elastic lamina (Fig. 27, I).

In areas where no plaques were seen, discrete swellings in the internal elastic lamina identical with the initial elastic lesions were noted. The media beneath the plaques was normal except for foci devoid of smooth muscle cells. These were occupied by collagen fibers. These foci were observed more frequently after prolonged cholesterol feeding than in animals fed for short periods of time.

Most of these alterations were similar to the chronic lesions reported by Buck [14] in the aortas of rabbits fed cholesterol.

Discussion

Striking alterations were observed in the endothelium and internal elastic lamina of coronary arteries in all 9 experimental rabbits. Of particular interest was the fact that lesions were noted following acute lipemia of only 24 hours' duration. This suggests that one should seek a mechanism by which administered lipids can produce pathologic changes very rapidly. Virchow's and Anitschkow's "imbibition" theory of atherosclerosis [27] meets this requirement. This holds that the lipid deposits are not products of degeneration of the arterial wall itself, but are derived from the plasma of the animal. The lipids were thought to be absorbed directly through the endothelium, along with nutrients. The fats were then believed to impregnate the ground substance of the arterial wall, following which there was a proliferation of the intima and cellular degenerative changes.

The dense bodies or caps observed by electron microscopy to be overlying endothelial cells were interpreted as complexes of lipid and protein adherent to the endothelial surface and undergoing a process of bit-by-bit imbibition or uptake. There are two reasons for suggesting

the possibility that this dense material may contain lipid. First, fat stains of coronary arteries from rabbits sacrificed during acute lipemia revealed numerous stained globules inside or in close proximity to the endothelial cells. Second, this substance is quite similar in density and texture to inclusions seen with the electron microscope within adipose cells found near the coronary vessels. It is likely that in addition to lipid, the dense endothelial caps may contain protein. Some of the protein may be fibrin or fibrinogen, as Duguid[28] has suggested. Removal of some of the lipid by solvent action would perhaps help to explain the occasional vacuoles seen within the dense clusters and the somewhat porous structure of these endothelial caps. The importance of lipoproteins in the pathogenesis of human and experimental atherosclerosis has been emphasized.[7, 29-31]

As mentioned previously, material similar to that in the dense caps was found in indentations of the plasma membrane, in vesicles, and in deep cavernous structures immediately beneath the accumulations. One may speculate, in accordance with the hypothesis of Virchow and Anitschkow, that this enclosed dense material represents lipid in the process of imbibition, uptake and transport. One can go further, and consider that the mechanism of uptake and transport may be that proposed by Palade,[32] Bennett,[33, 34] Moore and Ruska,[13] Fawcett and Selby[35] and others. Briefly stated, it is thought that the lipid and protein substances become bound to the lumen surface of the inner endothelial cell membrane. The cell responds by causing invaginations of the membrane at the points of binding, producing a cavity or "*caveola intracellularis.*" Further invagination leads to a pinching off of the cavity, leaving a vesicle within the endothelial cytoplasm. These structures have been observed as constant features of endothelial cells by Bennett, Luft and Hampton[36] and others.[13, 32-35] The vesicle is thought to be transported with its contents to the under surface of the endothelial cell where it fuses with the cell membrane, opens to the exterior and discharges its contents into the subendothelial space. This mechanism is similar to that which Lewis[37] has called "pinocytosis." Chapman-Andresen and Holter[38] and Chapman-Andresen and Prescott[39] have described the same process. Recently, Hampton[40] and Alksne[41] have found that colloidal mercuric sulfide particles (30 to 250 Å in diameter) are carried across hepatic parenchymal cells and dermal capillary endothelial cells within vesicles. Moore and Ruska[13] have used the word "cytopempsis" to apply to this process in endothelium.

It is pertinent to cite Buck's[14] recent work on the electron microscopy of endothelial cells after cholesterol feeding. He has described the vesicles and *caveolae,* and stated that these often resembled elements of

the endoplasmic reticulum. With cholesterol feeding, large cisternas containing "electron-scattering material" were noted in the endoplasmic reticulum. This material, he suggested, could be lipoprotein. He concluded by postulating that the small vesicles might be converted to endoplasmic cisternas, and thus constitute a pathway by which lipoprotein might enter the cells. However, Buck [14] did not postulate any mechanism for carrying this cholesterol to the subendothelial region.

Characteristic alterations in the internal elastic lamina were also seen after only 24 hours of lipemia. These very early initial elastic lesions, characterized by decrease in density and billowing of the elastica interna, with loss of its characteristic fibrillar nature, have not been described previously. It is interesting that they were first discovered with the electron microscope, having escaped detection with the light microscope.

However, the elastic components of the arterial wall have been implicated in the development of atherosclerosis by a number of authors. Anitschkow [2] and Wilens [42] made the point that the internal elastic lamella acted as a barrier, with the result that the lipoid masses were arrested at its surface for a considerable period of time after traversing the endothelium. The present investigation confirms these observations and extends them to rabbits only briefly subjected to acute lipemia. Frozen sections stained for lipid disclosed fatty material on and near the internal elastic lamina, but very little was found in the tunica media.

It is pertinent to note an observation made by Altschul.[43] He described deep indentations in the endothelial nuclei in experimental atherosclerosis and suggested that they might be due to the "impression of the (unstained) elastic fibers against the endothelial cell." In the present studies the initial elastic lesions were often seen to encroach upon the endothelial cells.

Holle [44] noted that occasionally in some areas of human arteries the first change seen in atherosclerosis was a swollen appearance which led to a diminished affinity for elastic tissue stains. Hass [45] reported that in xanthomatous cutaneous lesions, specifically in pseudoxanthoma elasticum, the elastic fibers in the middle and deep corium showed swelling, proliferation and fragmentation. Similar staining defects were seen in the internal elastic lamina with the light microscope in the present study, and were correlated with swelling, decrease in density and fragmentation of the elastica interna, as observed in the electron microscope. This suggests that similar pathologic alterations of elastic tissue may occur in various disease states which share the features of hypercholesterolemia or hyperlipemia. One wonders if the early swelling of the subendothelial ground substance described by a number of workers [6, 15, 16] might in reality represent swellings of the internal elastic lamina.

One is tempted to conjecture upon the relationship of the endothelial caps of dense material to the swellings noted in the internal elastic lamina. Lipid derived from these dense caps, after imbibition and transport across the endothelial cell by the mechanisms discussed above, would find itself, upon discharge into the subendothelial region, in close contact with the elastica interna. The accompanying rapid alterations in density, dimensions, fibrillation and staining reaction of the elastic tissue betray a correspondingly rapid change in its internal molecular constitution. This can be explained most logically by assuming that one or more lipid components, derived originally from the plasma, enter into solid solution in the internal elastic lamina. This is reasonable to expect in view of the high proportion of nonpolar amino acids in elastin. Moreover, the insertion of lipid or other foreign molecules between the protein chains of the elastic lamina can be expected to modify the elastic properties of the structure. If the solute molecules increased the degree of cross linking, increased brittleness would result. According to this view, the elastic tissue could be regarded as behaving like the stationary phase of a chromatographic paper or column, and the lipid would correspond to the moving phase. The lipid is "chromatogrammed" into the elastic tissue. A selectivity in such movements is to be expected, so that some lipid molecules would be taken in by the elastic protein in preference to others. This selectivity would depend on the molecular configuration of lipid and of elastic protein.

The initial elastic lesions as seen in electron micrographs are less dense than the elastic tissue itself and considerably less dense than the caps of material deposited on the lumen surfaces of the endothelial cells. One cannot readily distinguish how much of the density of a given object in an electron micrograph is due to bound osmium and how much is due to "native" or original tissue density. These factors make it difficult to interpret the significance of the relative densities seen in the electron micrographs. Yet, it is possible that the lessened density of the initial elastic lesion, as compared to that of the native elastin itself, may be due to the invasion of the elastic protein by a less dense component. However, uncertainties about amounts of bound osmium and possible selective extraction of lipids by solvents render any chemical interpretation of relative densities very precarious.

Turning now to more advanced lesions, we found in rabbit 9 characteristic plaques similar to those encountered by many previous workers. This rabbit had been subjected to chronic cholesterolemia from a cholesterol-rich diet administered for 3 months. In these plaques, the intra- and extracellular vacuoles seen with the electron microscope would seem to correspond to the globules of fat observed in the frozen sections with the light microscope.

A large number of the cells within the plaques appear to be smooth muscle cells. Malyschew [46] noted smooth muscle cells in plaques produced in experimentally ligated arteries. Anitschkow [2] and Altschul [4] described such cells in experimental atherosclerosis. These authors suggested that the cells migrated from the media to the intima. Altschul pointed out further that many of the smooth muscle cells entering the intima in experimental atherosclerosis contained cytoplasmic vacuoles and suggested that some might develop into typical foam cells. Some of the cells within the plaques bore no resemblance to smooth muscle cells. It would be pointless, with the limited knowledge now available, to conjecture further upon the nature and significance of these elements.

Summary

The ultrastructure of the alterations in the coronary arteries of 6 rabbits subjected to cholesterol feeding for various periods of time has been described and correlated with the alterations observed with the light microscope in 3 additional rabbits. In all 9 animals, striking alterations were seen in endothelial cells and in the internal elastic lamina. Such changes occurred even in rabbits sacrificed after acute lipemia of only 24 hours' duration.

Frozen sections stained for lipid, from animals sacrificed during acute lipemia, revealed lipid globules upon, within, and under the endothelial cells. With the electron microscope, clusters or caps of dense material were observed overlying portions of the lumen surfaces of a few endothelial cells. Similar dense material was found in numerous indentations of the plasma membrane, in vesicles, and in branching cavernous structures extending into the endothelial cytoplasm immediately beneath the dense endothelial caps.

Characteristic alterations were observed with the electron microscope in the internal elastic lamina of all the rabbits given cholesterol, including those sacrificed during acute lipemia. These "initial elastic lesions" appeared to be very early lesions since they were found after only brief (24 hours) acute lipemia. They appeared as discrete focal areas in the internal elastic lamina and were characterized by decrease in density and by swelling of the elastica interna with loss of its characteristic submicroscopic fibrillar nature.

Sections of tissue obtained from rabbits sacrificed during acute lipemia and stained for elastic tissue revealed discrete defects in the staining quality along the internal elastic lamina. These probably corresponded to the initial elastic lesions seen with the electron microscope. Deposits of lipid material along the internal elastic lamina were seen in frozen sections, but it could not be determined if the defects in elastic tissue staining corresponded to the deposits.

The dense caps overlying endothelial cells were interpreted as representing complexes of lipid and protein adherent to the endothelial surface and undergoing a process of bit-by-bit imbibition or uptake in accordance with the mechanism of transport suggested by Palade,[32] Bennett,[33] and Moore and Ruska.[13]

The initial elastic lesions were interpreted as representing a rapid change in the internal molecular structure of the elastica interna. This was most logically explained by assuming that one or more lipid substances from the endothelial caps were transported across the endothelium and brought into contact with the internal elastic lamina. The lipids were then envisioned as entering the elastic protein, forming a solid solution. Focal regions of such encroachment were apparent as the initial elastic lesions.

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[*Illustrations follow*]

Legends for Figures

All figures except 1, 2, 4, 5, 10 to 13, 26 and 27 are electron micrographs of rabbit arteries, fixed in buffered osmium tetroxide.

Key: A, endothelial cell
B, basement membrane
C, collagen (or reticular) fibers
E, elastic fibers
EC, endothelial caps
ES, initial elastic lesion
G, endoplasmic reticulum
H, fenestra in internal elastic lamina
I (or IEL), internal elastic lamina
K, mitochondria
L, lumen
M, myofilaments
N, nucleus
P, lipid
Q, defects in elastic tissue stain along the internal elastic lamina
R, thickened areas along internal elastic lamina
S, smooth muscle cells
TA, tunica adventitia
TI, tunica interna
TM, tunica media
U, vesicles
V, vacuoles
W, deep branching cavernous structures extending into endothelial cells
X, *caveolae intracellulares*
Y, cell membrane
Z, plaque

Fig. 1. A 3-dimensional diagram of the normal rabbit coronary artery. The tunica intima (TI) displays endothelial cells with nucleus, nucleolus, mitochondria and endoplasmic reticulum. These cells also contain numerous small vesicles and *caveolae*. The internal elastic lamina (IEL) is represented as a finely fibrillar structure of fairly even thickness, sending branching strands into the musculature. The tunica media (TM) contains many smooth muscle cells, and the tunica adventitia (TA) is represented as exhibiting fibroblasts, collagenous and elastic fibers and capillaries. The block cut from the artery shows the differences between longitudinal and transverse sections through the various coats. Many of the finer details shown in this illustration are drawn from the electron micrographs to follow. (Reproduced from Parker.[19] Used with permission of the publisher.)

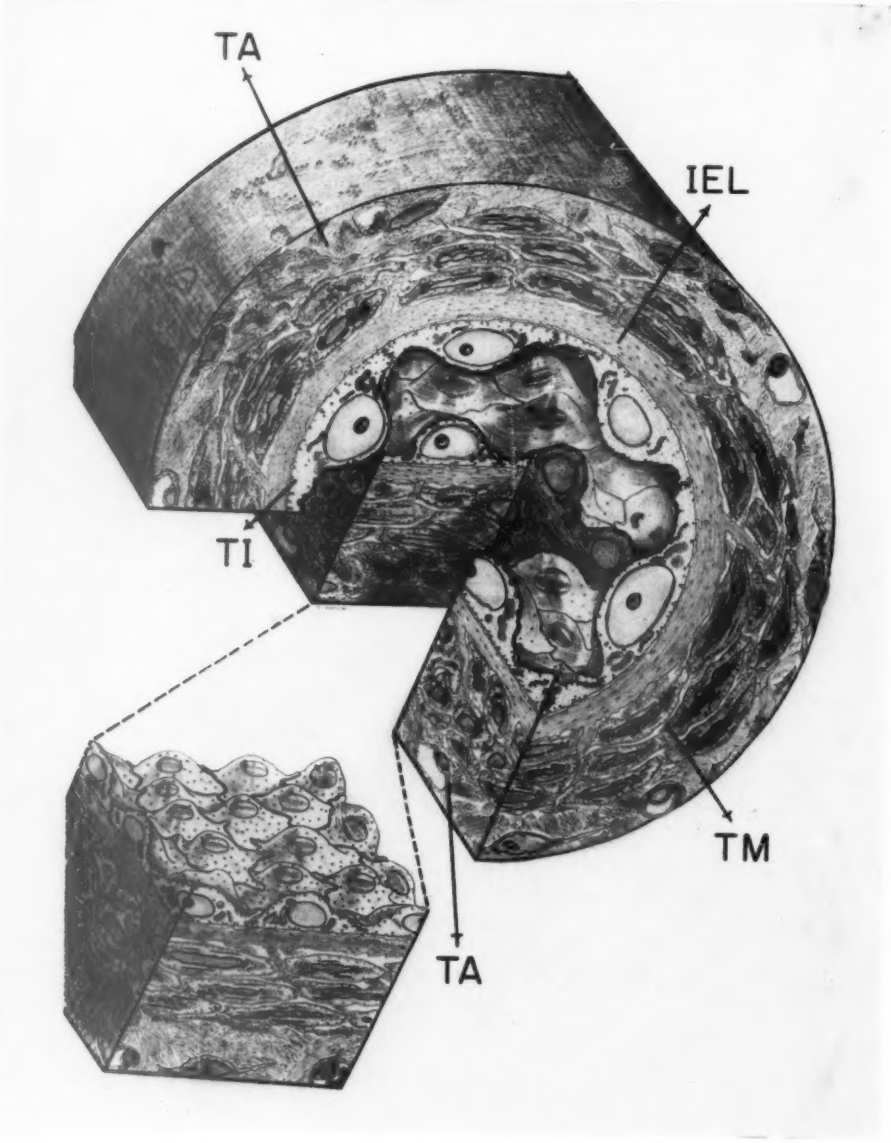

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FIG. 2. A 3-dimensional reconstruction of the rabbit coronary artery after the administration of cholesterol for short periods of time. The endothelial cell shows an accumulation of lipid-containing material on its lumen surface (EC). In the block cut from the main arterial segment, some of this dark lipoidal material is represented in *caveolae* and vesicles in the endothelial cell, in passage toward the internal elastic lamina. The latter displays several focal initial elastic lesions (ES), characterized by swelling, loss of density and loss of fibrillar texture. These lesions are attributed to lipid which has been selectively imbibed or "chromatogrammed" into the elastic tissue after traversing the endothelial cells in small vesicles. At this early stage after cholesterol administration no plaques are found.

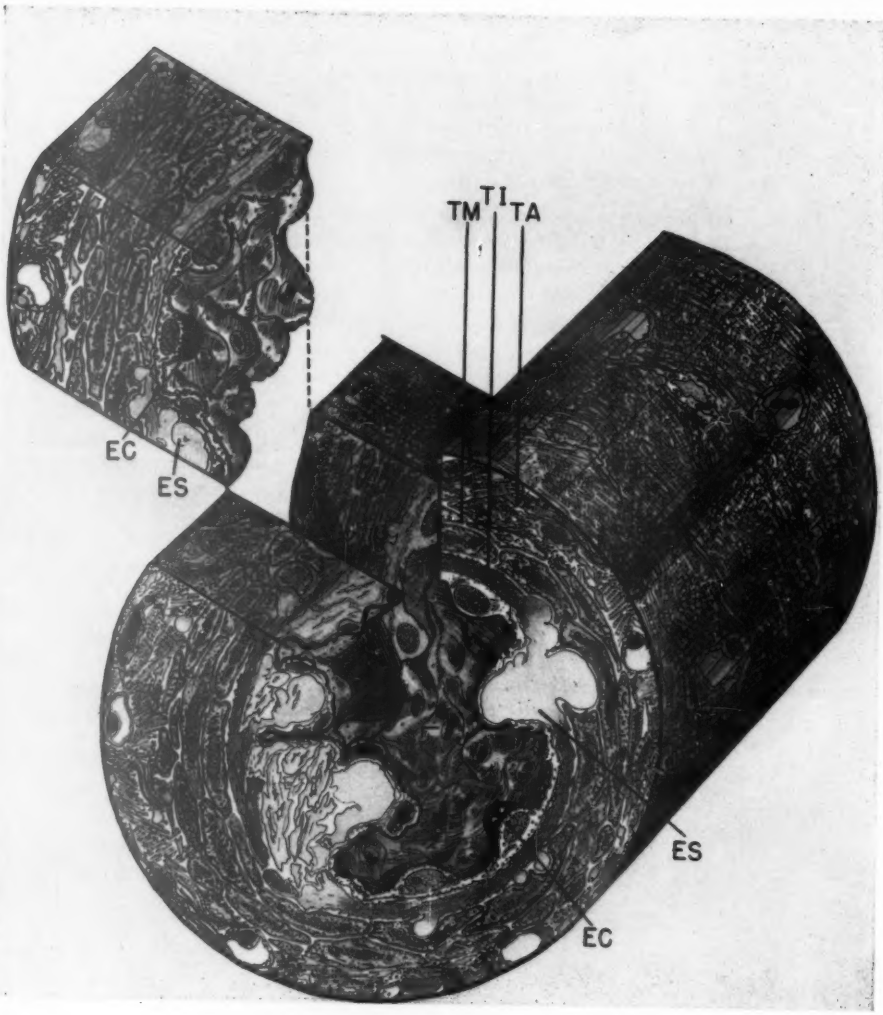

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FIG. 3. A cross section through a normal rabbit coronary artery, showing the relationship of the intima, media and adventitia. Note the ovoid profiles of endothelial cells (A) forming a continuous sheet resting upon the internal elastic lamina (I). The smooth muscle cells (S) are seen as fusiform profiles. Between the cells of the tunica media are small amounts of collagenous reticular fibers (C) and elastic fibers (E). × 3,000.

FIG. 4. A frozen section of coronary artery from rabbit 7, stained for lipid. Several small stained lipid globules (P) are seen on and within the endothelial cells. This rabbit received cholesterol for only 48 hours prior to sacrifice. Oil blue N stain; hematoxylin and eosin counterstain. × 1,800.

FIG. 5. A frozen section of coronary artery from rabbit 8, stained for lipid. Numerous globules (P) are observed on and in the endothelial cells and near the internal elastic lamina (I). Little lipid is seen in the media (TM). This animal was sacrificed after 48 hours of acute lipemia; there was no chronic cholesterol feeding. Oil blue N stain. × 1,200.

FIG. 6. An endothelial cell showing a cap consisting of a cluster of dense material (EC). Note that the material is inhomogeneous and appears somewhat porous. Also of interest are deep branching cavernous structures (W) seen extending deep into the endothelial cytoplasm (A), immediately beneath the endothelial caps (EC). These structures can be seen to contain similar dense material. Numerous vesicles (U) and *caveolae intracellulares* (X) containing similar dense material can also be seen within the cytoplasm. This section was taken from rabbit 5. × 20,000.

FIG. 7. An endothelial cell in rabbit 2. A dense endothelial cap is seen (EC). Note the numerous indentations of the plasma membrane immediately beneath the cap (X). These indentations contain similar dense material. × 7,000.

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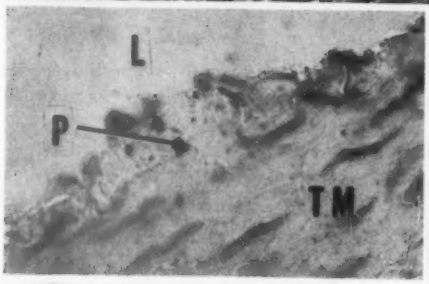

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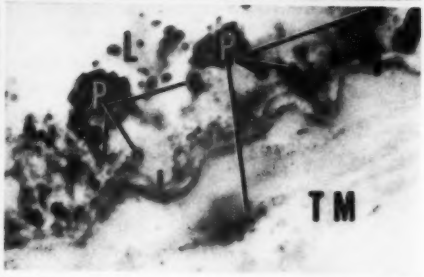

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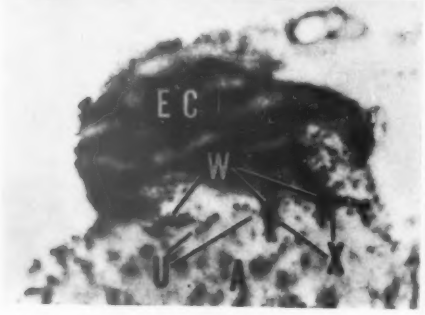

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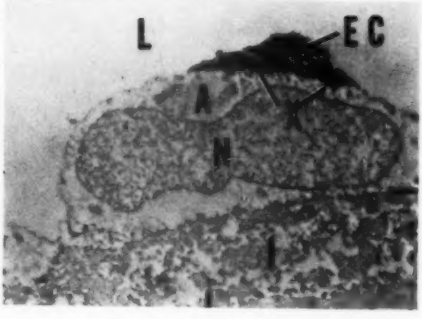

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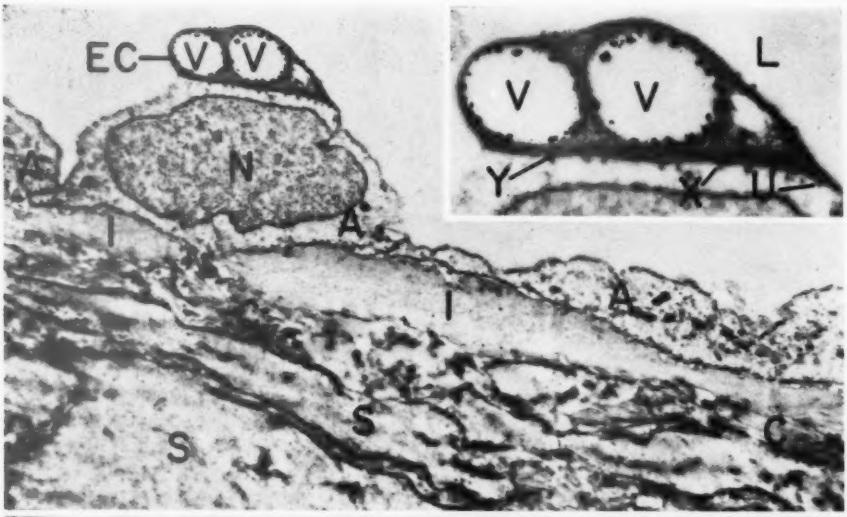
FIG. 8. An endothelial cap (EC) contains two ovoid vacuoles (V) with dense, irregularly shaped particles ringing them. Rabbit 1. × 6,000.

FIG. 9. A closer view of the endothelial cap (EC) pictured in Figure 8. Note the porous structure of the dense material and the numerous indentations of the plasma membrane (X) and vesicles (U) beneath the cluster. These contain dense material resembling that composing the endothelial cap. The cytoplasmic membrane (Y) of the endothelial cell is intact and discretely resolvable. × 13,000.

FIG. 10. A paraffin section through the intima and a portion of the media in rabbit 10, which was fed no cholesterol. The internal elastic lamina (I) is clearly seen. Orcinol-new fuchsin stain. × 1,600.

FIG. 11. A cross section through a coronary artery in rabbit 7, sacrificed after 48 hours of acute lipemia. Discrete focal defects (Q) in the staining quality of the internal elastic lamina can be seen. Orcinol-new fuchsin stain. × 1,200.

FIGS. 12 and 13. Portions of coronary arteries from rabbits 7 and 8 respectively. These animals were sacrificed following a 48-hour period of acute lipemia. Note the areas (R) of elastica interna which appear thicker than the normal lamina (I) and which stain less intensely. These are thought to be the initial elastic lesions. Orcinol-new fuchsin stain; hematoxylin and eosin counterstain. Figure 12: × 600. Figure 13: × 1,200.



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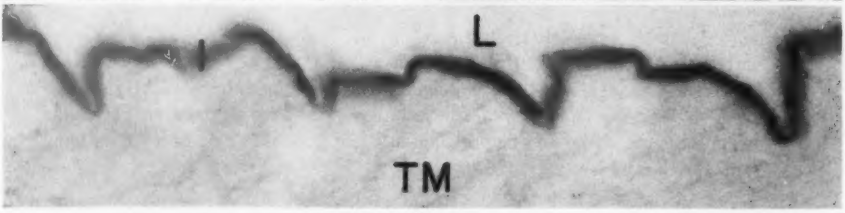

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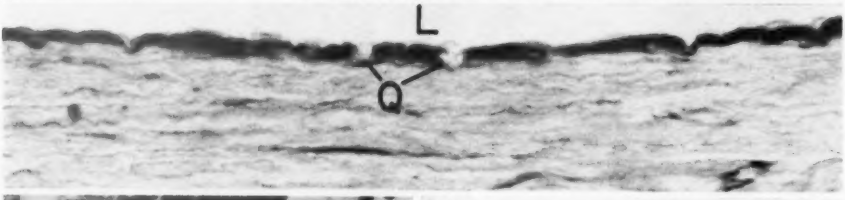

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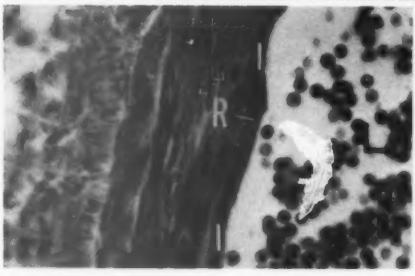

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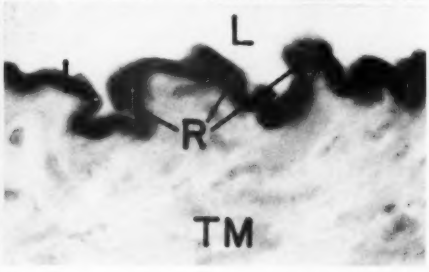

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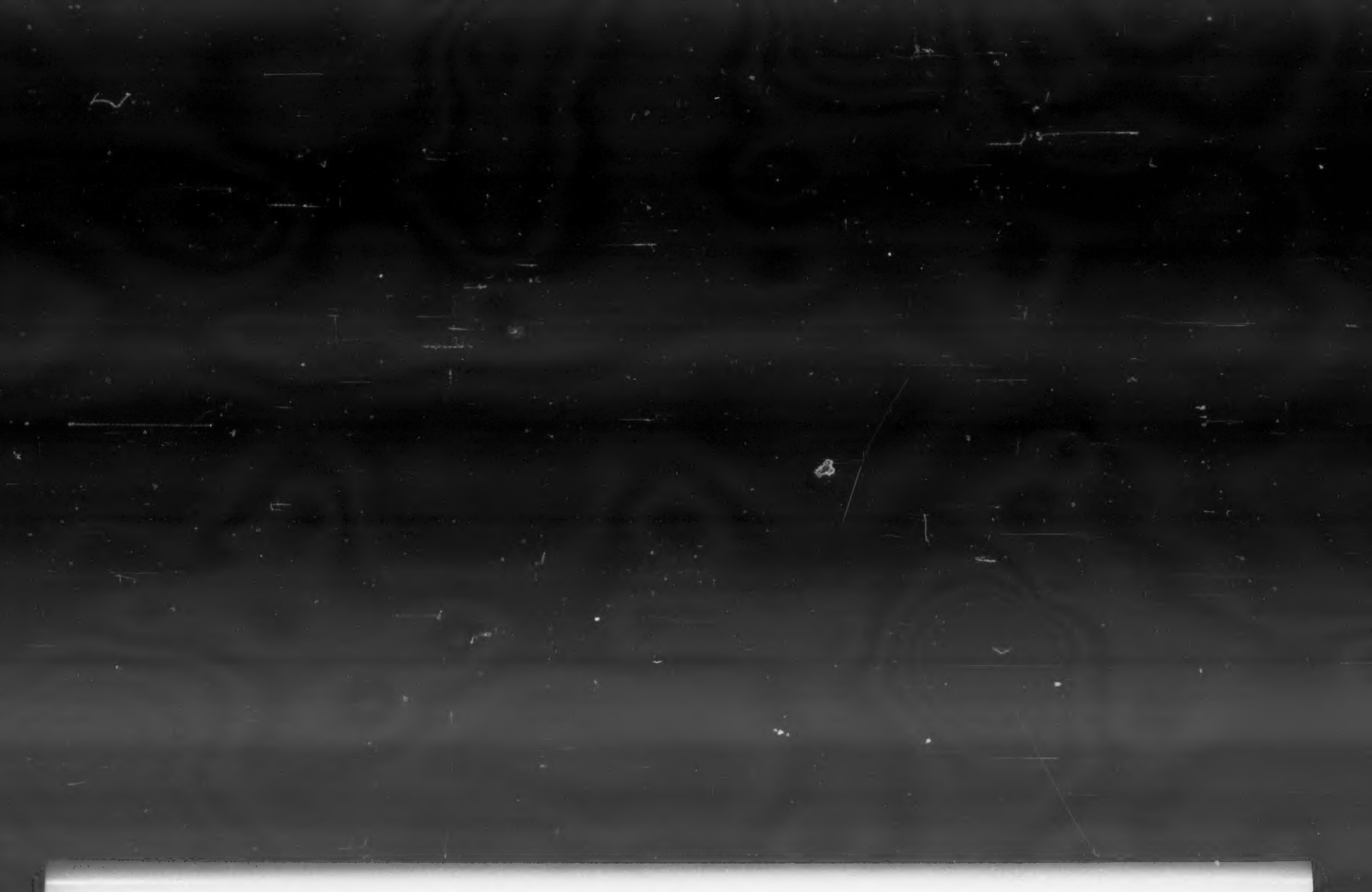
FIG. 14. A section showing the tunica intima and a portion of the tunica media (TM) from rabbit 1, sacrificed after 24 hours of acute lipemia. An initial elastic lesion (ES) is seen protruding from the lumen side of the internal elastic lamina (I) which otherwise appears relatively normal. The cytoplasm of an endothelial cell (A) is displaced by this swelling. × 6,000.

FIG. 15. A view of the initial elastic lesion pictured in Figure 14, at higher magnification. × 10,000.

FIG. 16. A more severe initial elastic lesion (ES) in rabbit 2. Normal-appearing internal elastic lamina (I) merges with an area which is characterized by a decrease in density, and there is bulging of the elastica interna with loss of its characteristic fibrillar nature. The initial elastic lesion is seen to cause the endothelial cell cytoplasm to protrude into the lumen. It also encroaches upon the smooth muscle cells (S). × 4,000.

FIG. 17. The initial elastic lesion pictured in Figure 16 at higher magnification. × 6,000.



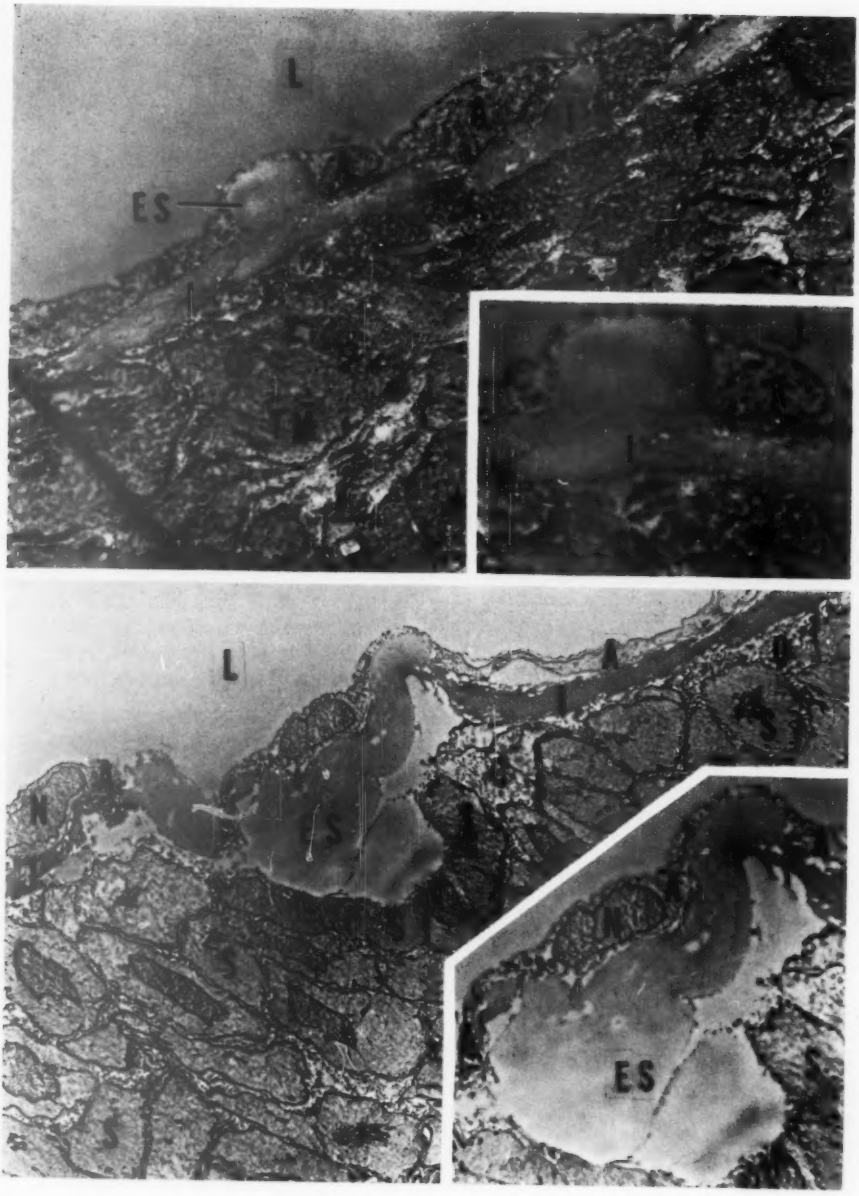


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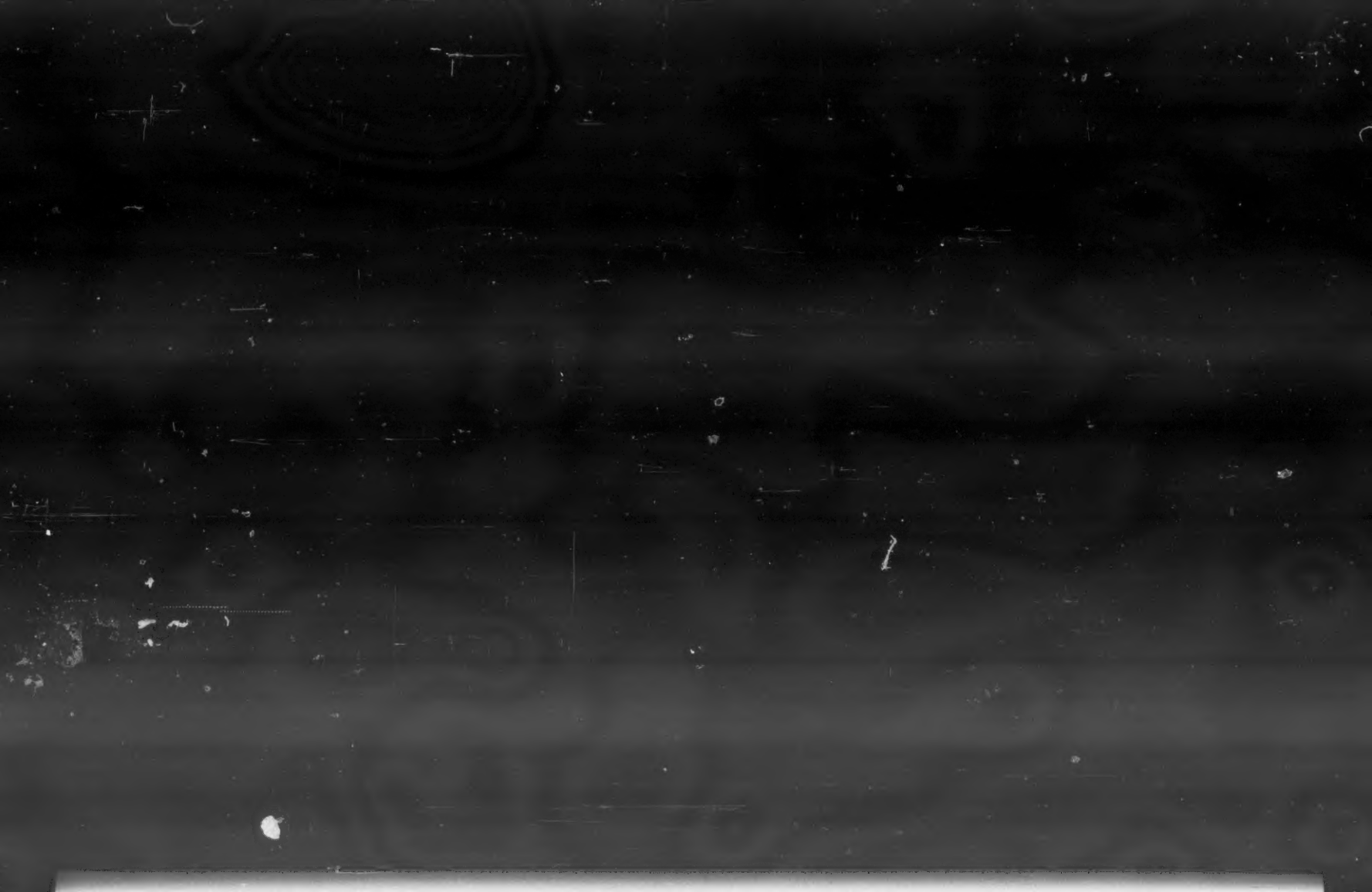
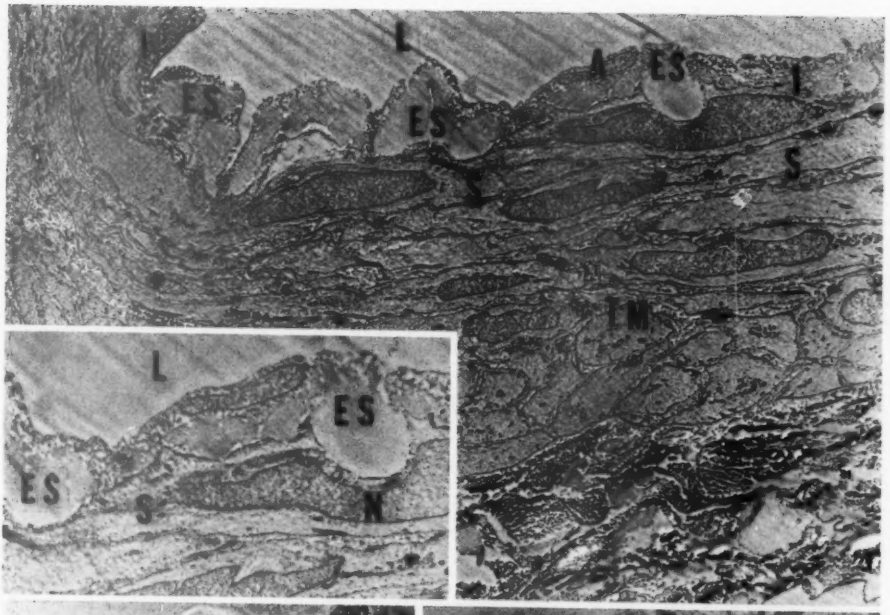
FIG. 18. A section from rabbit 5, which was given acute lipemia for 48 hours by intravenous administration of cholesterol. Numerous initial elastic lesions (ES) are seen along the internal elastic lamina (I). × 4,000.

FIG. 19. A higher power view of one of the initial elastic lesions (ES) pictured in Figure 18. Note how the billowed elastic tissue encroaches upon and deforms the nucleus of a smooth muscle cell (N). × 4,500.

FIG. 20. A coronary artery in rabbit 3, which was sacrificed after 24 hours of acute lipemia. Note the large initial elastic lesion (ES) in the upper portion of the photograph. This protrudes far into the lumen (L) of the vessel. In the lower portion of this figure there is a large ovoid defect within the cytoplasm of an endothelial cell (arrow). The defect contains material which resembles that characteristically found in initial elastic lesions. It is interpreted as representing a tangential section through a bulge of an adjacent elastic lesion. × 3,000.

FIG. 21. A view of the endothelial cell described in Figure 20 at higher magnification. Note the fenestration (H) in the internal elastic lamina (I). Such fenestrae are also seen in normal arteries. × 10,000.





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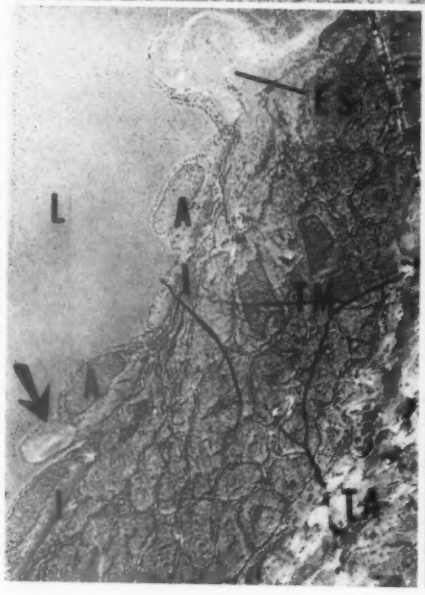

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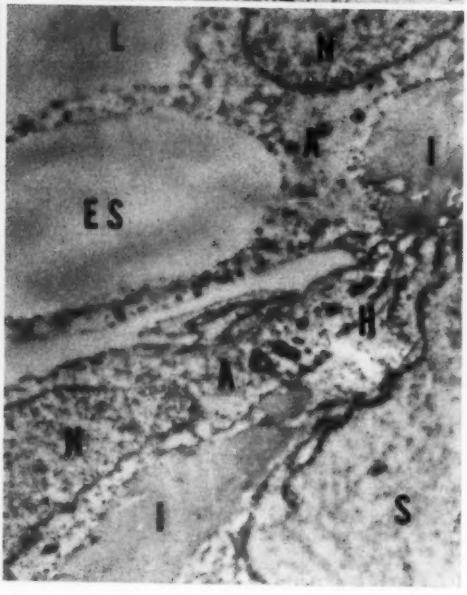

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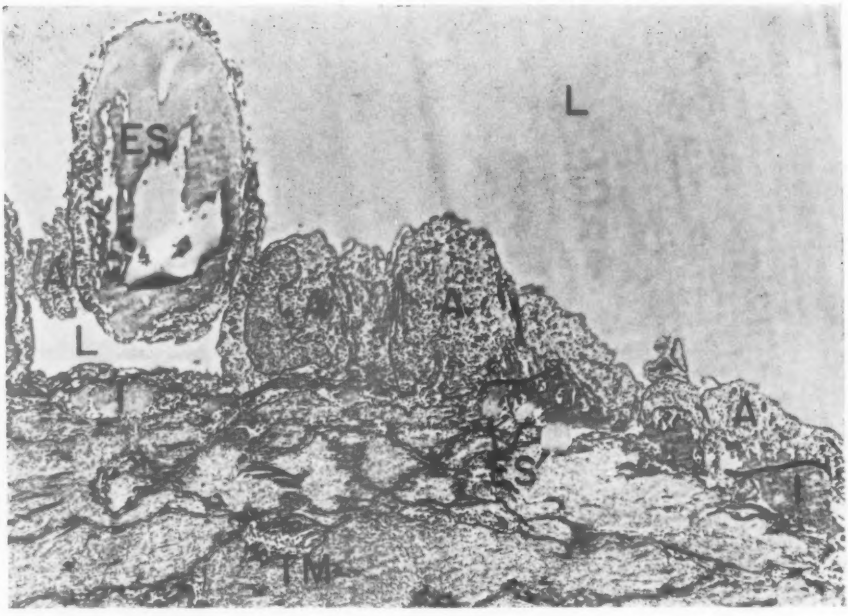

FIG. 22. An oblique section of the coronary artery. The large ovoid profile in the left upper portion of the photograph represents a section through the top portion of an initial elastic lesion (ES) which has pushed up under an endothelial cell (A). The elastic tissue is seen to be surrounded by endothelial cytoplasm. Note the small initial elastic lesions (ES). The tissue was obtained from rabbit 2, sacrificed after 48 hours of acute lipemia. × 4,000.

FIG. 23. A portion of the tunica media (TM) and tunica adventitia (TA) in rabbit 5, sacrificed following 48 hours of acute lipemia induced by intravenous administration of cholesterol. Portions of the elastic fibers (E) display lesions closely resembling those of the internal elastic lamina. × 4,000.

FIG. 24. A full thickness of a coronary vessel wall. There is an increased amount of collagen (C) between the smooth muscle cells (S). Rabbit 5, fed cholesterol for 48 hours. × 3,000.

FIG. 25. The perinuclear region of a smooth muscle cell (S) from the tunica media of a coronary artery in rabbit 3, sacrificed following 24 hours of acute lipemia. The cell contains a large ovoid vesicle (arrow) with an irregular dense border and 2 or 3 lamellas running diagonally across it. Numerous mitochondria (K) and vesicles (U) are also present. × 23,000.

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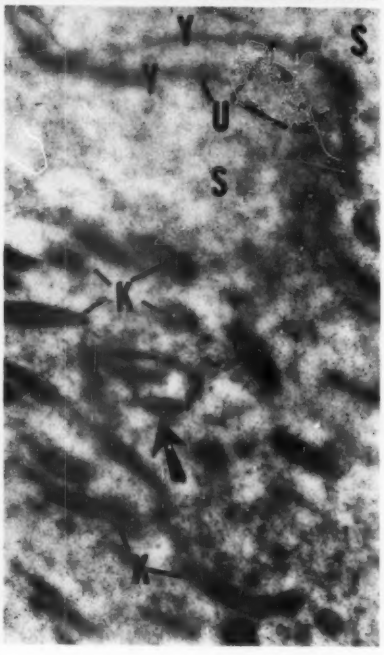

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FIG. 26. A frozen section stained for lipid depicting typical experimental atherosclerotic plaques (Z) in rabbit 9, fed cholesterol for 104 days. Lipid (P) can be seen throughout the plaques. Oil blue N stain. × 150.

FIG. 27. A section obtained from rabbit 9 and stained for elastic tissue. Small elastic fibers (E) are seen within the plaque (Z). The internal elastic lamina (I) appears to be intact and shows no obvious changes except for displacement. Orcinol-new fuchsin stain. × 1,200.

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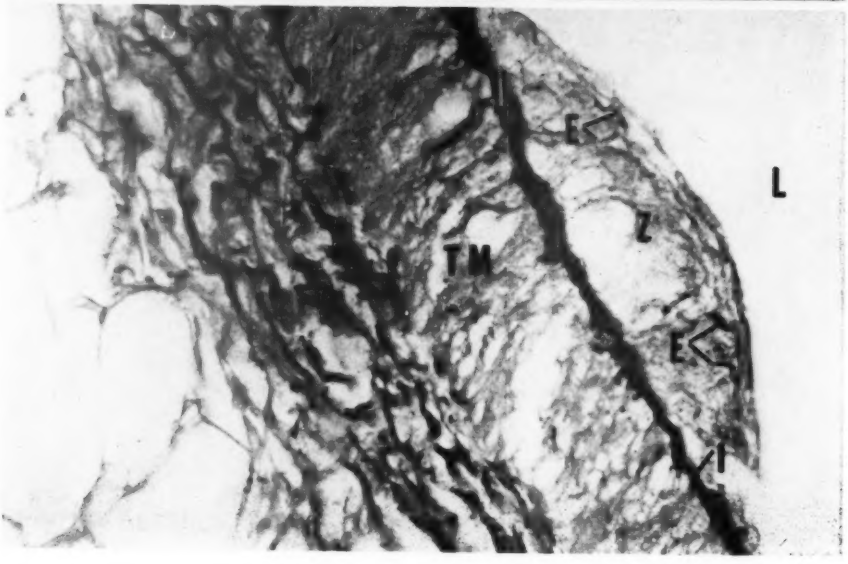

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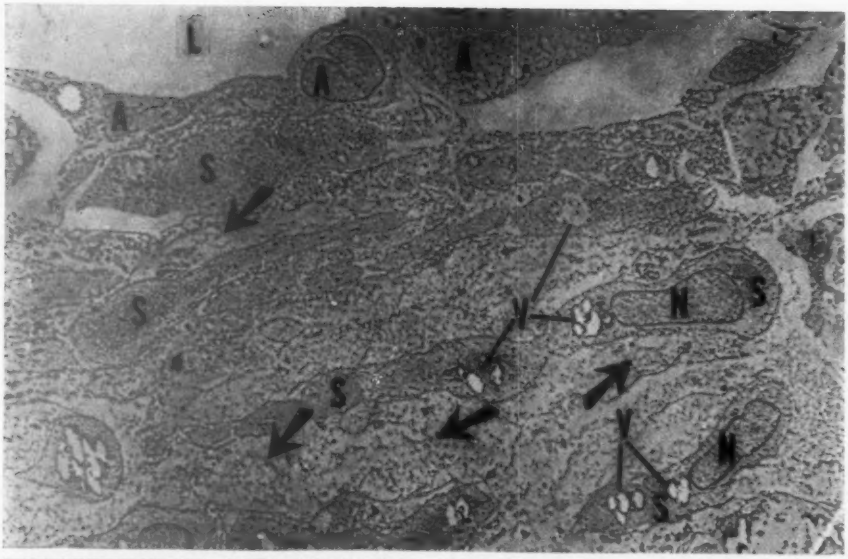
FIG. 28. The top of an atherosclerotic plaque in rabbit 6, fed cholesterol for 104 days. The endothelial cells (A) contain many small vacuoles (V). There are numerous vacuolated cells with elongated profiles beneath the endothelium. These are thought to be smooth muscle cells (S). No internal elastic lamina is seen. The extracellular spaces contain dense, small particles (arrows) which often surround areas devoid of particles. The intracellular vacuoles and the extracellular rounded areas devoid of particles are thought to represent sites of lipid corresponding to the stained areas in Figure 26. × 8,500.

FIG. 29. One of the cells resembling smooth muscle seen within a plaque. The vacuoles (V) presumably contained lipid prior to fixation. There is an elongated nucleus (N) with endoplasmic reticulum (G) and mitochondria (K) in the perinuclear region. A basement membrane (B) surrounds the cell. Rabbit 6. × 7,000.

FIG. 30. A plaque from a coronary artery in rabbit 6. The elongated cell in the upper left corner of the photograph contains many vacuoles, presumably lipid. × 3,000.



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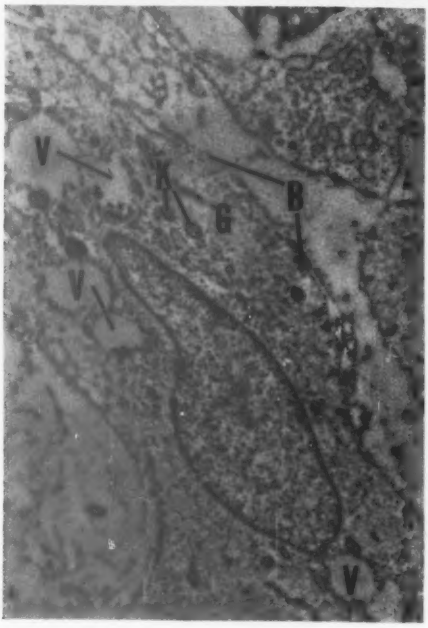

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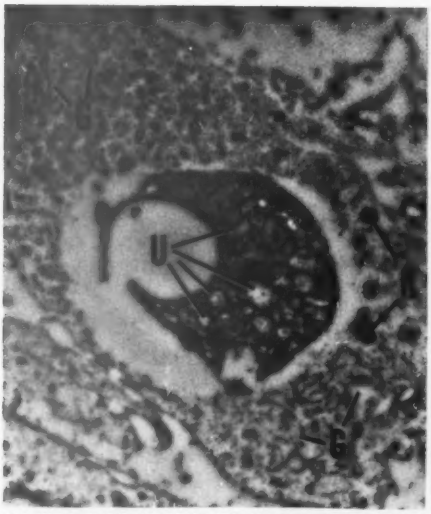
FIG. 31. An unusual type of cell found within a plaque. Note the endoplasmic reticulum (G) and mitochondria (K). Most conspicuous is a large, somewhat ovoid, dense area within the cytoplasm, containing numerous irregularly shaped vacuoles and vesicles (U). Rabbit 6. × 10,000.

FIG. 32. An area near the periphery of a plaque in rabbit 6. The endothelial cells (A) are intact. There is no definitive internal elastic lamina, but only small fragments of elastic tissue (E). An increase in the extracellular elastic tissue (E) is noted between the smooth muscle cells (S). × 3,000.

FIG. 33. A portion of a plaque near its edge, in rabbit 6. The endothelial cells (A) contain cytoplasmic vacuoles (V). Small fragments of elastic tissue (E) are seen beneath the endothelial cells. A number of smooth muscle cells (S) are found beneath these fragments. A wide sheet of elastic tissue is interposed between smooth muscle cells. This is believed to be the internal elastic lamina (I). × 7,000.

FIG. 34. Endothelial cells in a section from rabbit 6. Numerous large vacuoles (V) can be seen within the cytoplasm. It is possible that these vacuoles may represent lipid inclusions resembling those shown in Figure 26. × 8,500.



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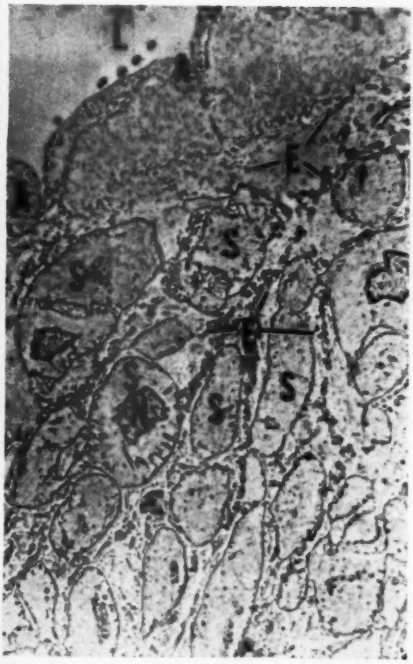

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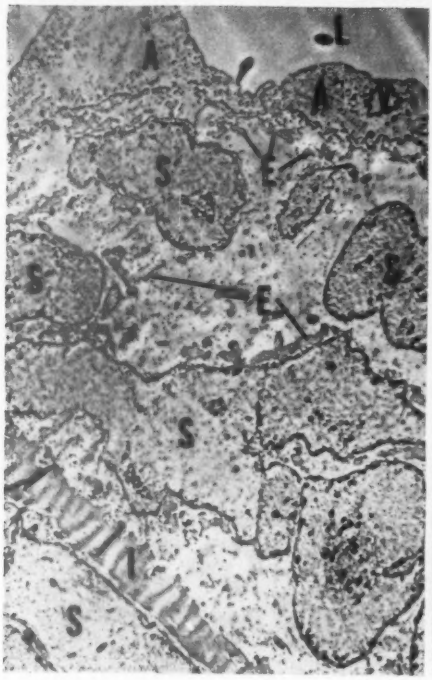

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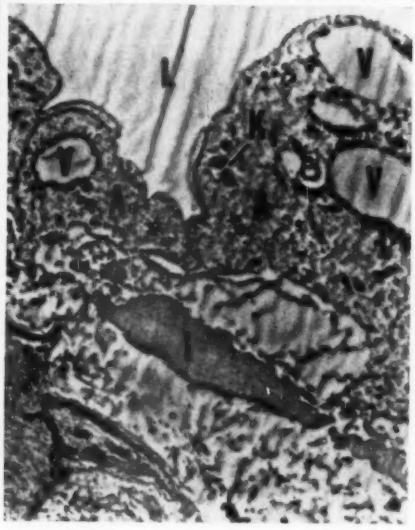

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HISTOCHEMICAL OBSERVATIONS ON GLYCOGEN IN THE HUMAN MYOCARDIUM

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Early investigations of stainable glycogen in the postmortem myocardium of adult man led to a divergence of results and opinions.[1] Quantitatively, glycogen was variously considered to be dependent on such factors as nutritional status, freshness of the tissues, methods of fixation and processing, cardiac chamber examined, and fiber function (conductional or nonspecialized). However, in the heart of the fetus and newborn, it was generally conceded that much glycogen was usually present.[1] In infants up to the age of 8 months, Mowry and Bangle[2] found that many hearts contained considerable glycogen, irrespective of age, cause of death, cardiac weight, or postmortem interval.

In patients with diabetes not treated with insulin, Warren[3] found glycogen to be absent from myocardial infarcts but persisting in high concentrations in the surrounding viable myocardium. Comparing insulin-treated cases with nondiabetic controls, he noted little difference in total glycogen content of the heart. On the other hand, Mowry and Bangle[2] concluded that irrespective of therapy, diabetes appeared to promote the accumulation of glycogen even in portions of the myocardium remote from an infarct. In fact, they considered extensive glycogen content of the myocardium confirmatory evidence of diabetes. Excessive glycogen deposit in the hearts of infants born to diabetic mothers has also been claimed.[4, 5]

Investigation in animals has shown that in the myocardium, the normally rich supply of glycogen[6-13] becomes markedly depleted subsequent to coronary artery ligation,[6-8] fulminating systemic anoxia,[6, 9-11] and postmortem autolysis.[6, 7, 11, 14] Depletion of glycogen after coronary artery ligation proceeded at a rapid rate. In the rat, glycogen disappeared from the ischemic region within 5 to 15 minutes,[6] and in the dog, glycogen depletion was observed in 30 minutes.[7, 8] In respect to systemic anoxia, the rate of depletion in the beating heart of rats was of the same order of magnitude as that observed in the ischemic area deprived of its blood supply by coronary artery ligation.[6] By comparison, depletion due to

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postmortem autolysis was far slower in the rat[6] and also, according to Kent,[14] in the dog. Only the data of Yokoyama and his co-workers[7] indicated that the rates of glycogen depletion in the canine myocardium were similar in coronary artery ligation and postmortem autolysis.

In other experiments, the quantity of myocardial glycogen could be modified by epinephrine,[10, 15] norepinephrine,[15] insulin,[9, 16] cardiac glycosides,[17] hyperthyroidism[18] and severe anemia.[18] Increased concentrations were induced by fasting,[9, 19] administration of growth hormone,[19, 20] or pancreatectomy.[16]

The study reported here deals with the heart of human adults. It was intended originally to investigate the histochemical characterization of focal myocytolysis, a metabolic lesion of the myocardium frequently observed in arteriosclerotic heart disease.[21] Because this lesion was found to be rich in glycogen,[22] it was considered desirable to reinvestigate the relation between myocardial ischemia and glycogen content and also to evaluate other conditions thought to influence the glycogen content of the myocardium. The experiments in the rat, cited above, had been designed to obtain information on this subject under controlled conditions.[6]

Material and Methods

The hearts of 41 unselected human adults were examined. There were 22 males and 19 females, 20 to 86 years of age (median age, 61 years).

After dissection of the coronary arteries, the hearts were opened, cleansed of postmortem blood clot, and weighed. The myocardium was examined by numerous parallel incisions. A cardiac "map" of each specimen was prepared to serve as a pictorial and topographic record of coronary arteriosclerosis and myocardial abnormality, and also to note the sites from which tissue blocks were taken for microscopic examination. From 3 to 8 blocks (average 5) were selected in each case, to include the whole thickness of the cardiac wall and to represent both normal and abnormal myocardium. Samples were taken from the free walls of the left and right ventricles in all cases, from the interventricular septum in all but 3 cases, and from the pectinate portion of the right atrium in all but 5 cases. The left atrium samples were obtained only in the last 6 cases.

In order to investigate the effects of prolonged autolysis, 26 specimens chosen at random from among the 41 cases were stored in a closed container at 4° to 8° C.; additional blocks were taken from the left ventricle 9 to 105 hours after the initial sampling. Usually, the additional blocks were from the myocardium contiguous to the sites originally sampled.

The tissues were fixed in modified Carnoy's solution (3 parts absolute alcohol, 1 part glacial acetic acid) at room temperature for 12 to 36 hours. They were then washed in toluene for 2 hours and processed for paraffin embedding. Serial sections of each block were stained as follows: hematoxylin and eosin (H and E); the periodic acid-Schiff (PAS) method of McManus,[23] without counterstain; the PAS method preceded by digestion with diastase, at pH 7.2; and the PAS method with hematoxylin counterstain.

Sections stained with H and E were used to identify pathologic alterations such as infarction, hydrops and myocytolysis.[21] Glycogen was quantitated in PAS-stained sections, taking into account its concentration per unit area and its regional distribution in the left ventricle and interventricular septum, on a scale of 0 to 4 (trace to 1+, poor, slight, mild; 2+, moderate; 3+ and 4+, rich, intense, marked) (Fig. 1). Glycogen was identified by its PAS staining characteristics and by its digestibility with diastase. While the method was semiquantitative and the differentiation between adjacent grades admittedly subjective, larger differences were unequivocal. The quantitation was complemented by correlating the distribution of glycogen with anatomic landmarks and pathologic features.

Each case was characterized with respect to the following: (a) cardiac abnormality; (b) postmortem interval prior to examination; (c) nutritional state; (d) diabetes mellitus; (e) blood glucose; (f) hemoglobin concentration; (g) pulmonary abnormality; (h) congestive heart failure; (i) angina pectoris; (j) essential hypertension; (k) shock; (l) serum potassium and urea nitrogen; (m) digitalis therapy.

Coronary arteriosclerosis was graded as severe (including occlusions), moderate, mild or absent. Cardiac hypertrophy was considered present if the heart weight was in excess of 400 gm. in men, and 350 gm. in women. The diagnosis of myocardial infarction was based on histologic verification in H and E stained sections, and included fresh, healing and healed stages. The designation of healing as against fresh infarct was based on the demonstration of a repair zone, however narrow.

The hearts which served as controls fulfilled the following: (a) no more than mild coronary arteriosclerosis; (b) weight within normal limits; (c) normal valvular apparatus; and (d) normal myocardium.

Results

General Observations

As recorded in Table I, glycogen deposit in the left ventricle was correlated with the presence or absence of infarction, severe coronary arteriosclerosis, cardiac hypertrophy, and valvular disease. Among the

9 control hearts, glycogen content was no more than 1+, with a single exception of 2+. Cardiac hypertrophy, valvular disease or severe coronary arteriosclerosis were not associated with significant alteration of glycogen distribution from that noted in the control hearts. However, with myocardial infarction, 14 of 19 hearts were found to be the seat

TABLE I

GLYCOGEN IN THE MYOCARDIUM, LEFT VENTRICLE AND INTERVENTRICULAR SEPTUM

	Severe coronary arteriosclerosis	Hypertrophy	Valvular disease	Stainable glycogen: Slight (0 to 1+)	Moderate (2+)	Marked (3+ and 4+)
Control						
(9 hearts)	0	0	0	8	1	0
No infarct						
(13 hearts)	+	0	0	3	0	0
	0	+	0	4	0	1
	0	0	+	2	0	0
	0	+	+	2	1	0
Infarct						
(19 hearts)	+	0	0	1	1	2
	+	+	0	1	1	10
	+	+	+	1	0	2

of rich glycogenization. The age of the infarct appeared to be of major importance since intense glycogen deposits were observed in all 6 hearts containing fresh infarcts, in 6 of 8 hearts in which infarct was healing, and in 2 of 5 hearts in which it was healed.

Histologic Features

Control Hearts. When present at all, glycogen was limited to a few muscle fibers in the subendocardial zone.

Infarct. Regardless of duration, the area of infarction was invariably devoid of glycogen (Fig. 2) as were the surrounding granulation tissue and the scar which replaced it (Fig. 3). However, throughout the viable myocardium surrounding the fresh infarct, glycogen deposit was widespread (Fig. 2). Even when infarction was limited to the subcavitary portion of the wall, glycogen extended at times to the epicardium. There was a distinct topographical gradient, the glycogen concentration being greatest immediately adjacent to the infarct (Fig. 2). With aging and repair, the area of glycogen deposit became reduced to a narrow and often discontinuous collar surrounding the infarct (Fig. 3). In the case of healed infarcts, such glycogen as was present had a patchy distribution and failed to display any topographical preference for the myocardium about the infarct. Distributed throughout small infarcts but restricted to the borders of those which were submassive, the larger interfascicular

septums were often bordered by cuffs of viable myocardium with rich glycogen content. This phenomenon recapitulated, in miniature, the situation in the zone surrounding infarcts.

Either preceding the appearance of leukocytic infiltration in fresh infarcts or simultaneous with it, necrotic glycogen-free muscle again became PAS-positive. This reaction, however, was neither particulate nor digestible with diastase (Fig. 3). As in the case of glycogen, this reaction could be blocked by omitting the oxidation step in the PAS procedure. The necrotic fibers remained PAS-positive until they were disposed of by reparative processes.

As a rule, necrotic PAS-positive muscle in fresh infarcts was bordered directly by viable myocardium containing glycogen. However, in some instances there was an intervening narrow zone which lacked both glycogen and nondigestible PAS-positive substance. The scar tissue which replaced necrotic muscle was PAS-positive in a manner identical with that of necrotic muscle.

Neutrophils accumulating in response to acute infarction contained glycogen. In all probability this was not the result of phagocytosis of cardiac glycogen since fresh infarcts were depleted of glycogen hours before leukocytes made their first appearance (Fig. 2). Moreover, glycogen is a normal constituent of leukocytes.[24]

Hydrops and Myocytolysis. Hydrops of muscle fibers and myocytolysis have been viewed as progressive stages occupying positions intermediate between the normal state and necrosis[21] (Text-fig. 1). These alterations (Figs. 3 to 5) appeared either as miliary foci in otherwise normal myocardium or in zonal fashion about an infarct. Affected muscle fibers were ordinarily rich in glycogen, regardless of location. Glycogen deposit usually extended somewhat beyond the region exhibiting myocytolysis (Fig. 5).

The glycogen content of the specialized Purkinje fibers has been the subject of investigation in a variety of animals[13] and in man.[1] These fibers, which characteristically present a hydropic appearance, are located in the subendocardial region and, depending on species, have been said to contain more, less, or equal amounts of glycogen as compared with the nonspecialized myocardium. In the series of human hearts we have examined, the subendocardial zone was devoid of glycogen in the normal specimens; this zone was commonly rich in glycogen in ventricles (left) which were the seat of infarction, especially in the vicinity of the infarct (Fig. 4). While this observation cannot be construed as evidence for or against the existence of Purkinje fibers in the human heart, it does indicate that neither the hydropic appearance nor glycogen deposit is peculiar to the conduction system.

Structure of Stainable Glycogen. The histologic pattern of stainable glycogen was variable. At one extreme glycogen appeared as fine particles aligned with and applied to the cross striations of the myofibers. This distribution was obscured beneath the natural surfaces of the heart because of intracellular drifting of glycogen, an artifact of fixation. At the other extreme, glycogen appeared as very coarse particles approximately the size of red blood cells but lacking orientation as to cell structure. The coarse particles, particularly prevalent in hydropic fibers and those undergoing lysis, occupied the spaces which appeared empty in H and E stained sections. PAS-positive and diastase-digestible granules were also found in the stroma, especially in regions of high glycogen concentration. It could not be ascertained whether this represented glycogen liberated from muscle fibers, an independent accumulation of glycogen in the stroma, or a postmorten artifact.

Glycogen in Chambers Other Than the Left Ventricle. In the 9 control hearts, the right ventricle and atrium contained little or no glycogen and thus resembled the left ventricle. Among the remaining 32 cases there were 4 with rich glycogen deposit in both the right ventricle and atrium, and 6 in which only one or the other had a rich glycogen content. With one exception, in all hearts in which the right ventricle contained large amounts of glycogen, there was also much glycogen in the left ventricle, incident to infarction. However, there was an equal number of hearts with infarction and rich glycogen deposits in the left ventricle in which little glycogen was found in the right ventricle. Thus, any parallelism of abundant glycogen deposit between left and right ventricles appeared fortuitous. Analysis of individual cases revealed that marked glycogen deposit in the right ventricle was associated with and probably dependent upon the coexistence of infarction in this very chamber, either as miliary foci or by extension of a massive left ventricular infarction. The one exception was a case of severe mitral stenosis in which the right ventricle was rich in glycogen though the left ventricle was not. The meaning of this is unclear in that two other cases with severe mitral stenosis failed to exhibit this disparity of glycogen content.

Glycogen deposit in the right atrium, as in the right ventricle, was associated with much glycogen in the left ventricle in all but one case. Unlike the right ventricle, however, abundant glycogen in the right atrium was associated with local infarction in only 3 of the 7 cases. No reason for this divergence was apparent. In the 6 cases from which samples were obtained from both the left and right atriums, there was a clear parallelism in the amount of glycogen on the two sides. Both atriums contained equal amounts in 2 hearts; in the remaining 4, glycogen appeared in neither atrium.

Postmortem Autolysis

The interval between time of death and tissue fixation ranged from 2 to 61 hours, with a median of 6½ hours. Of the hearts placed in fixative within 6½ hours, 52 per cent were rich in glycogen in contrast to only 20 per cent of those placed in fixative after more than 6½ hours. However, the influence of postmortem interval was more apparent than real; all hearts with fresh infarcts were rich in glycogen even though 50 per cent were fixed later than 6½ hours, and all but one of the control hearts contained little glycogen even though 75 per cent were fixed prior to 6½ hours. The prefixation interval after death for the anatomically normal hearts varied from 3 to 17 hours and for the hearts with infarcts from 2 to 27 hours. Thus, within the limits of the period of observation, the amount of glycogen present appeared to correlate more closely with the state of the myocardium than with the duration of postmortem autolysis.

Among 12 of the hearts rich in glycogen, which were resampled after 7 to 67 hours (median, 23 hours), 10 showed a decline in glycogen content of no more than 1+. Sampled once more after an additional 24 to 48 hours, 6 of 7 hearts still contained at least 1+ glycogen. Thus, depletion of myocardial glycogen by protracted postmortem autolysis progressed at a very slow rate.

Nutritional State

The nutritional state—i.e., "obesity-leanness" of the body—was determined by the thickness of the panniculus in the anterior abdominal wall. Measurements of the panniculus adiposus were available in 39 of the 41 patients. In Table II the cases are classed according to (a) thickness of panniculus; (b) glycogen in left ventricle and interventricular septum; and (c) presence or absence of myocardial infarction. Because of the small size of the sample, no subdivision by sex was made.

While hearts with little glycogen were found in haphazard distribution among the obese and the lean individuals, hearts with abundant glycogen were concentrated among the better nourished cases. This apparent correlation between nutritional state and rich glycogen content is clearly spurious and due to the greater frequency of myocardial infarction in the well nourished. Furthermore, with a single exception,* there was no case showing marked glycogen deposit without infarction and no fresh infarct unaccompanied by abundant glycogen. Similarly, the majority of cases with healing infarcts showed marked glycogen in the myo-

* This case was omitted from Table II because massive anasarca from renal failure precluded accurate measurement of the abdominal panniculus.

TABLE II

NUTRITIONAL STATE, MYOCARDIAL INFARCT, AND GLYCOGEN IN MYOCARDIUM

Panniculus adiposus (cm.)	No. cases	Stainable glycogen: Slight (0 and 1+)				Moderate (2+)				Marked (3+ and 4+)			
			Myocardial infarct				Myocardial infarct				Myocardial infarct		
		None	Healed	Healing	Fresh	None	Healed	Healing	Fresh	None	Healed	Healing	Fresh
< 1.0	5	3	1	0	0	0	0	0	0	0	0	1	0
1.0-1.9	3	1	0	1	0	0	0	0	0	0	0	1	0
2.0-2.9	6	4	0	0	0	1	1	0	0	0	0	0	0
3.0-3.9	14	6	0	0	0	0	0	0	0	0	1	1	6
4.0-4.9	5	1	1	0	0	1	0	1	0	0	0	1	0
5.0-5.9	4	2	0	0	0	0	0	0	0	0	0	2	0
6.0-6.9	1	1	0	0	0	0	0	0	0	0	0	0	0
7.0	1	0	0	0	0	0	0	0	0	0	1	0	0
Total	39	18	2	1	0	2	1	1	0	0	2	6	6

The difference in the amount of glycogen between the hearts with an infarct and those without an infarct is highly significant ($P << 0.01$) by the Chi square test.

cardium irrespective of the nutritional state. Among the cases with healed infarcts, the myocardium was rich in glycogen, if at all, only in the better nourished individuals with an abdominal panniculus of 3 cm. or more. Thus, the nutritional state of the body was only indirectly related to the glycogen content of the myocardium at necropsy.

Diabetes Mellitus

Of the 12 patients with diabetes in this series, the myocardium in 9 (75 per cent) revealed rich glycogen content. Because each of these 9 hearts was also the seat of infarction, and because 40 per cent of all hearts with abundant glycogen were from nondiabetic individuals, the glycogen content of the myocardium did not seem to be correlated with diabetes as such. Among the 3 hearts which contained little or no glycogen in patients with diabetes, one was in the control group, another had severe coronary arteriosclerosis without infarction, and the third contained a healed infarct. All the patients had had diabetes for several years, but none were known to be of the "juvenile" type. Since most of the patients had been critically ill with myocardial infarction, cancer, infection, etc., attempts at detailed evaluation of the diabetic status were unrewarding. Moreover, sufficient biochemical data during the terminal hours of life were unavailable.

Blood Glucose Concentration

In 5 diabetic patients no correlation was evident between the intensity of myocardial glycogen deposit and blood sugar level as determined 1 to 2 days prior to demise (range, 111 to 644 mg. per cent; median, 198). This was also true in those nondiabetic individuals made hyperglycemic transiently by intravenous administration of glucose solution. Agonal blood sugar levels were not available for correlation with the amount of glycogen in the myocardium at necropsy.

Anemia

Assuming that the abundance of glycogen observed in the myocardium surrounding areas of infarction, in foci of myocytolysis, and in hydropic muscle, is due to "relative" anoxia (in contrast to glycogen loss attributable to "absolute" anoxia in infarction), the possibility presents itself that severe anemia may influence the deposition of glycogen in the myocardium. Hemoglobin concentration had been determined in 36 of the 41 patients during the terminal period of hospitalization. Marked anemia (hemoglobin, 7 gm. per cent or less) was present in 8 patients (22 per cent), of whom 4 had abundant myocardial glycogen. Among the remaining 28 without severe anemia, the hearts were rich in glycogen in 8.

All but one of the 12 glycogen-rich hearts contained infarcts. Thus, no correlation between cardiac glycogen and anemia could be demonstrated. At the same time, such an association could not always be excluded. For example, the unexpected observation of 2+ glycogen in one anatomically normal heart may have been related to the patient's severe anemia (4 gm. per cent), since all other contributory influences were excluded. Similarly, the one exceptional case of marked glycogen content among the hearts without infarcts (Table I) had severe anemia.

Pulmonary Disease

The possibility that anoxia related to pulmonary disease might interfere with myocardial metabolism was considered. Pulmonary lesions encountered in this group of cases included, in order of frequency: (a) acute pulmonary edema, 39 per cent; (b) pneumonia, 29 per cent; (c) pulmonary arteriosclerosis, 27 per cent; (d) thromboembolism, 24 per cent; (e) senile emphysema, 24 per cent; (f) chronic passive congestion, 17 per cent; (g) bronchitis, 15 per cent; and (h) miscellaneous conditions (primary or secondary tumors, pulmonary fibrosis and thoracic vertebral deformity), 7 per cent each. No correlation could be recognized between glycogen content and either incidence or type of pulmonary abnormality.

Congestive Heart Failure

Congestive heart failure was present in 14 cases; it was acute in 6, chronic in 6, and of unknown duration in 2. Abundant glycogen was present in the myocardium in 7 of the 14 cases (50 per cent), and in 4 of 21 cases (19 per cent) which were not in failure. In the remaining 6 of the 41 cases, the status of cardiac compensation was unknown. Regardless of the existence or duration of congestive heart failure, all hearts rich in glycogen contained infarcts. Thus, no independent correlation between the degree of glycogen deposit and congestive heart failure could be demonstrated.

Angina Pectoris, Hypertension and Shock

The available data concerning angina pectoris, hypertension and shock were incomplete. However, the impression was gained that any association between these conditions and the amount of myocardial glycogen was mediated through the anatomic state of the myocardium; i.e., the presence or absence of infarction.

Serum Potassium and Blood Urea Nitrogen

In glycogen-rich zones abutting upon myocardial infarcts, Poppen, Green and Wrenn[25] demonstrated large amounts of potassium histo-

chemically. This and other evidence[26] indicated a close biochemical relationship between intracellular glycogen and potassium.

Serum potassium concentrations had been determined in 14 patients and were inferred from the serum levels of urea nitrogen in 26. There was intense myocardial glycogen deposit in 5 of 7 cases (71 per cent) with hyperkalemia (5.1 to 6.5 mEq.; median 5.8 mEq.) and in 1 of 7 patients (14 per cent) with serum potassium below 5 mEq. (2.5 to 4.5 mEq.; median, 4.3 mEq.). Similarly, the myocardium was rich in glycogen in 5 of 8 patients (63 per cent) with azotemia (urea nitrogen, 54 to 162 mg. per cent; median 84), but in only 5 of 18 patients (28 per cent) with little or no nitrogen retention (urea nitrogen, 11 to 44 mg. per cent; median, 24). Analysis of individual cases showed that these differences in glycogen coincided with, and almost certainly depended on the coexistence of myocardial infarction.

Cardiac Glycosides

Cardiac glycosides (usually digitalis preparations) had been administered to 10 patients for one week or more until the time of death whereas 6 others had received glycosides only during the last 2 to 3 days of life. In 5 of these 16 patients, digitalis effects were evident in electrocardiographic tracings. Cardiac glycosides appeared to have little if any influence upon the amount of myocardial glycogen. The hearts from patients given digitalis were about equally divided between those with much glycogen and those with little or no glycogen. Again, analysis of the individual cases indicated that abundant myocardial glycogen reflected the coexistence of myocardial infarction.

Discussion

Examination of the human heart for stainable glycogen at necropsy revealed the following: (1) Hearts considered anatomically normal contained little or no glycogen. (2) In the majority of the hearts with infarction, viable myocardium (in the left ventricle and interventricular septum) was rich in glycogen, often with a distinctive pattern of distribution related to the topography and age of the infarct. (3) Whether fresh, healing, or healed, myocardial infarcts were invariably devoid of glycogen. (4) Glycogen was abundant in and about foci of myocytolysis as well as in hydropic fibers.

Two major questions were raised by these observations: First, why is glycogen present only in small amount in the normal heart or absent from it? Second, why is it usually abundant in the abnormal heart?

The observation of a paucity of stainable glycogen in normal hearts after death is at variance with the evidence that during life the myo-

cardium of man * [27] and other mammals [6-13] is rich in glycogen.† From animal experiments it appeared that the two factors, body temperature and anoxic cardiac action, might account for postmortem depletion of glycogen. With respect to the former, myocardial glycogen was found to disappear in rat hearts within 2 hours when ablated ventricles were kept at room temperature.[6] In the dog the myocardium lost all stainable glycogen within 4, 5 or 24 hours when kept, respectively, at body (37° C.),[14] room (28° C.),[7] and refrigerator (4° C.) [14] temperature. Relative to anoxic cardiac action, there is suggestive evidence that under natural conditions glycogen depletion of the human myocardium is not so much the result of postmortem autolysis as of circulatory or respiratory failure during the agonal period. In the rat, for example, we have shown that such agonal events as apneic anoxia associated with unimpeded cardiac contraction result in a profound depletion of myocardial glycogen within a few minutes.[6]

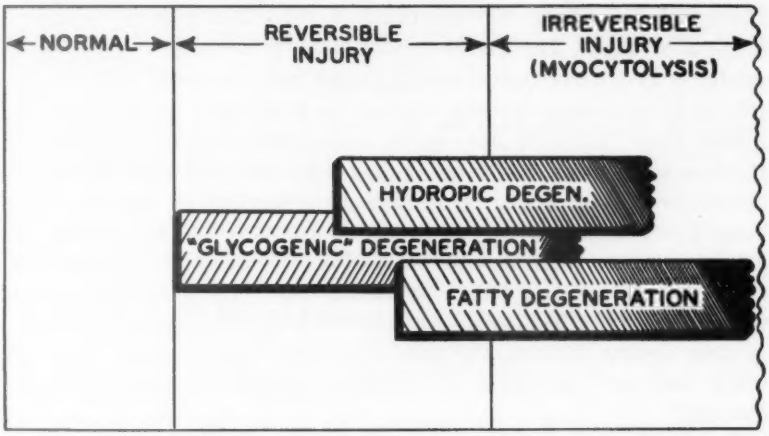
If it is assumed that glycogen depletion is the usual feature of normal human hearts examined at necropsy, the persistence of glycogen in quantity must be viewed as abnormal. Warren [8] and Yokoyama and coworkers,[7] examining hearts with infarcts, considered the phenomenon to denote an abnormal increase of glycogen. We submit that several other mechanisms operating singly or in combination may be adduced to explain the persistence of glycogen: (1) Glycogen may not have been utilized because of altered intracellular metabolism. (2) It may not have been consumed because of impaired muscular contraction. (3) It may have been present in an altered physicochemical state.

The first possibility carries with it the connotation of cellular injury and may explain the patterns of glycogen concentration in hearts with infarction: the wide distribution accompanying acute infarction; the progressive restriction to a narrow collar about the infarct upon healing; and the inconstant and haphazard focal distribution once the infarct has healed. From our knowledge of the circulatory complexities accompanying coronary arteriosclerosis of man in contrast to the single-point obstruction of coronary artery ligation in the experimental animal, one may presume that the impact of ischemia in human coronary arterial insufficiency will extend with lesser intensity beyond the confines of the region of infarction. In a previous publication,[21] infarction was viewed as but an extreme in a continuum of graded injuries affecting the metabolic integrity of the myocardium. As in parenchymatous, hydropic and fatty degenerations, the postmortem persistence of gly-

* Personal observations of left auricular appendages obtained during commissurotomy procedures in patients with mitral stenosis.

† Biopsy specimens were made available through the cooperation of Dr. Ralph Friedlander during experimental cardiac surgery in the dog.

cogen may, also, signify metabolic damage to the muscle cell, which might be termed "glycogenic" degeneration. The dynamic interrelations of these degenerative alterations are illustrated in Text-figure 1 and are corroborated by observing in a single representative focus the per-



TEXT-FIGURE 1. Concept of interrelation between various forms of myocardial degeneration. "Glycogenic," hydropic, and fatty degenerations may occur independently of each other; they may also coexist as in a focus of myocytolysis or even within the same fiber. These degenerative lesions are potentially reversible. However, increasing damage depicted by denser shading may become irreversible. The relative positions of "glycogenic," hydropic and fatty degeneration in the diagram are based on observations in the ischemic myocardium. For example, fatty degeneration is thought to require a more severe insult than "glycogenic" degeneration, inasmuch as the former is confined to the zone immediately contiguous with the infarct whereas the latter is widespread. The relative position of parenchymatous degeneration ("cloudy swelling") in the schema is undetermined.

sistence of glycogen in fibers which are hydropic, in those which contain stainable fat, and in those which appear essentially normal with conventional stains. "Glycogenic" degeneration is considered to be potentially reversible, paralleling the efficacy of collateral circulation. Suboptimal circulatory compensation will result in the persistence of glycogen or even lead to slow cell death within the areas of glycogen deposit, i.e., focal myocytolysis.

The second possibility—incomplete or faulty consumption of glycogen because of impaired muscular contraction—cannot be sharply separated from "glycogen" degeneration and, in fact, may merely constitute another facet of it. The interdependence of muscular contraction and glycogen content has been demonstrated in the rat heart beating under conditions of acute and fulminating anoxia. When muscular contraction was arrested instantaneously, the rapid rate of glycogen depletion which was characteristic of anoxic contraction was slowed and exhibited the

gradual disappearance characteristic of postmortem autolysis.[6] By inference, glycogenized muscle such as that found surrounding the infarct may be considered to have contracted less vigorously than normal myocardium.

As to the third possibility, an altered physicochemical state of the glycogen, reference may be made to the existence of at least two different forms of glycogen. This has been postulated on the basis of differential solubility in trichloroacetic acid.[28] It has been observed that the insoluble form is not only more resistant to generalized anoxia than the soluble form[11] but also more stable in the border zone of an infarct.[29] It is conceivable that variations in the results recorded of chemical and histochemical analyses of myocardial glycogen may be related to the tissue fixatives utilized. These may permit part of the glycogen to be lost into solution prior to application of histochemical techniques. It is also conceivable that physicochemical alterations of glycogen increase its thermostability in relation to the effects of body temperature and postmortem cooling. Finally, the possibility exists that glycogen variants which are not susceptible to available autolytic enzymes may be formed.[30]

Summary

An investigation of the hearts from 41 adults has been conducted to elucidate the significance of stainable glycogen in postmortem myocardium. Although normal myocardium is apparently rich in glycogen during life, little or none is detected after death. Glycogen depletion is not considered to be a mere postmortem phenomenon. Experiments in the rat indicate that it may be initiated by agonal cardiorespiratory failure and, indeed, be completed during the process of dying.

Persistence of glycogen was observed in 15 of the 41 hearts. It was considered to be a pathologic feature since it was found almost exclusively in association with myocardial infarction. The difference in the amount of glycogen in hearts with infarcts and those without was highly significant by the Chi square test. The infarcts themselves were invariably devoid of glycogen.

The following parameters appeared to have no independent bearing upon the persistence of glycogen: postmortem interval, function of fibers (conductional versus nonconductional), nutritional status, diabetes mellitus, pulmonary disorder, congestive heart failure, hyperpotassemia, or administration of cardiac glycosides. There was some suggestion that severe anemia (hemoglobin below 7 gm. per cent) might occasionally be contributory.

The distribution of glycogen in the viable portions of the left ventricle

was related to the topography and age of the infarct. Accompanying the fresh infarct, glycogen was distributed widely; upon healing, it became progressively limited to a rather narrow zone bordering the infarct. These dynamic shifts and the very fact of glycogen persistence were considered indicative of metabolic myocardial injury. The concept of "glycogenic" degeneration is proposed to indicate kinship with other forms of metabolic disorder, i.e., parenchymatous, hydropic, and fatty degenerations.

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The authors acknowledge the assistance of R. R. Freudenthal, Ph.D., in the statistical analysis of Table II.

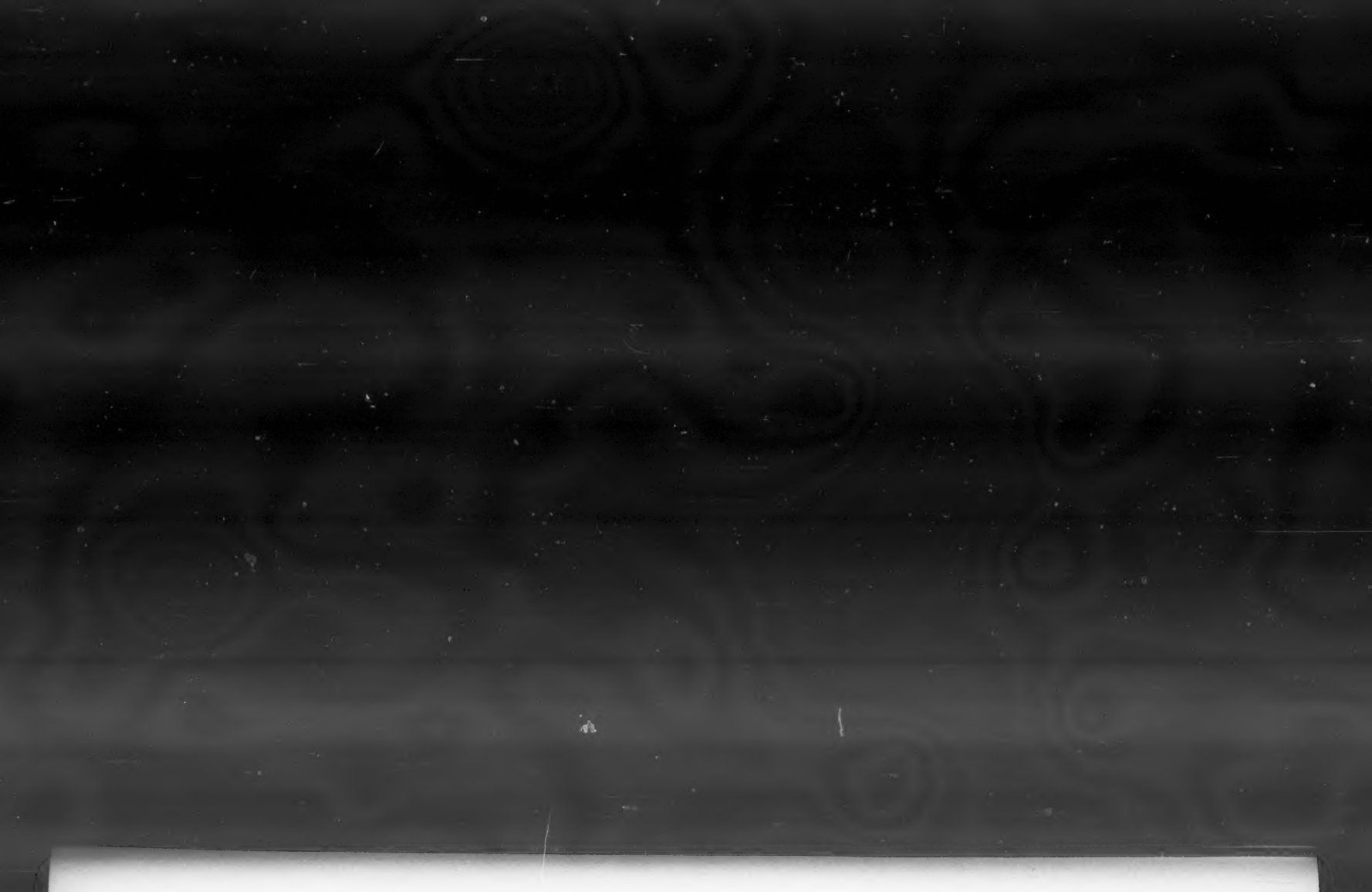
[*Illustrations follow*]

Legends for Figures

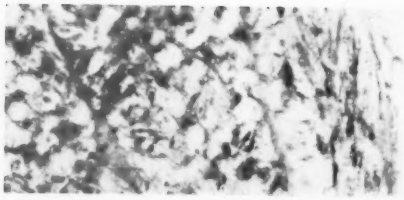
Fig. 1. Quantitation of glycogen. Figure 1A illustrates 1+ and Figure 1B illustrates 4+ glycogen. Periodic acid-Schiff stain without counterstain or application of diastase. X 140.

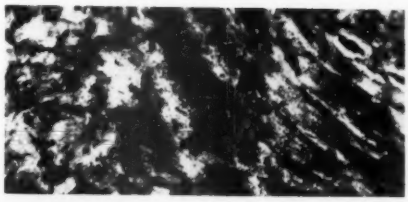
Fig. 2. Acute myocardial infarction. In this and subsequent photographs, A and B are adjacent sections stained respectively with hematoxylin and eosin and PAS. No diastase digestion is employed in the latter. In Figure 2A the infarct is very fresh. An acute inflammatory reaction has not evolved in the field illustrated but was found elsewhere in the section. The infarct in Figure 2B is devoid of glycogen but bordered by a broad zone rich in glycogen, declining in concentration from above downward, i.e., toward the epicardium. X 56.

Fig. 3. Viable myocardium surrounding a healing infarct and showing a collar of glycogen deposit, low-grade hydropic degeneration and myocytolysis. The reparative tissue at the top of the photograph is faintly PAS-positive. The reaction which is not due to glycogen is best appreciated by comparing its dark gray shade with the light gray of the myocardium at the bottom of the photograph, from which glycogen was absent. X 140.



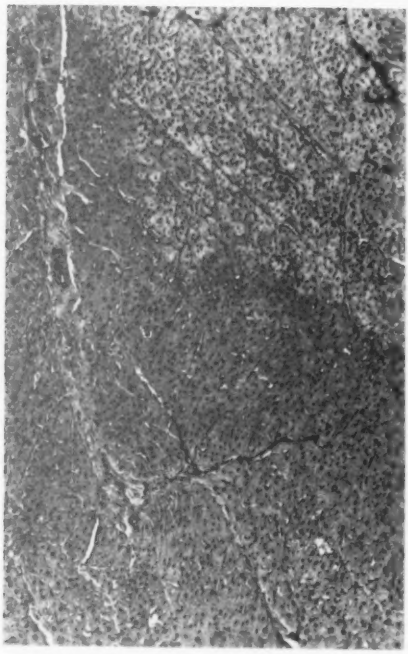
1A





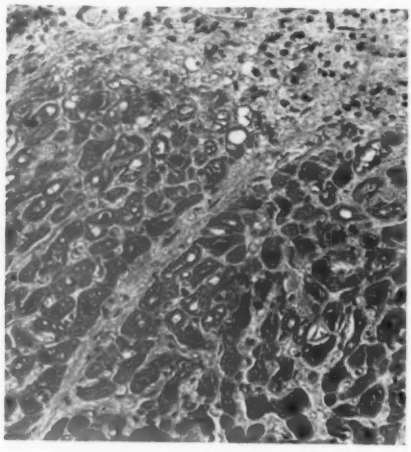
1B

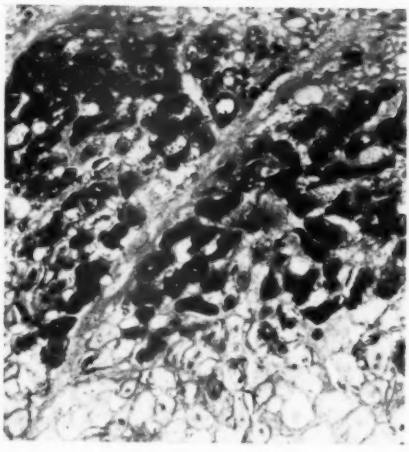
2A



2B

3A





3B

FIG. 4. Subendocardial myocardium, showing hydropic degeneration. Note the particulate nature of the glycogen and its greater density in the nonvacuolated portions of the fibers. The glycogen-free wedge at the bottom of the photograph is scar tissue. X 140.

FIG. 5. Focus of early myocytolysis involving a small group of fibers (circle). The focus lies in a larger field characterized by persistence of glycogen. Except for a few hydropic fibers, most of the glycogen-rich myocardium is structurally unaltered. The focus is topographically unrelated to an infarct. X 180.

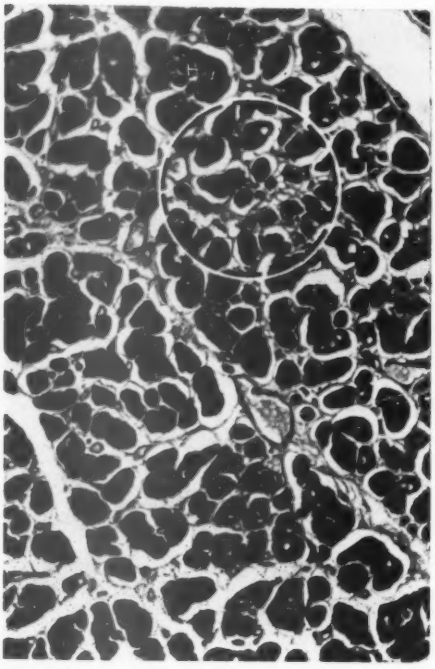
4A

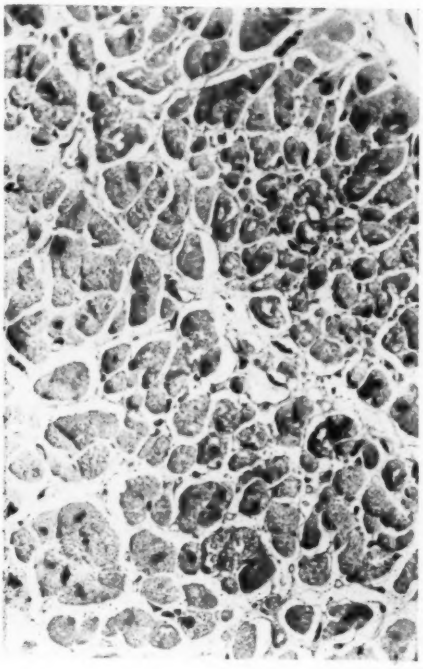
5A

4A

4B

5A





5B

THE EFFECTS UPON THE KIDNEY OF TRANSIENT HYPERCALCEMIA INDUCED BY PARATHYROID EXTRACT

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Many investigators have described renal lesions in association with hypercalcemia under both experimental and clinical conditions.[1-15] There has been no unanimity of opinion, however, as to their nature and location. Tubular degenerative changes, characterized by fatty and hyaline droplet formation, calcification and necrosis of epithelium, cellular proliferation, and the formation of casts of various types have been noted. Interstitial calcification and fibrosis have been emphasized by some observers [6, 9, 10]; others have noted the calcification to be restricted to the tubules and basement membranes.[1, 2, 4, 7, 8, 15] Dilatation of tubules, often filled with calcific masses, and tubular atrophy have been described.[1, 6, 8, 10, 15] Calcification of glomeruli and renal arteries have also been noted on occasion.[2, 4, 7, 10] With respect to localization of these lesions, there has been no agreement. Either localization has not been attempted, or the alterations have been found in all parts of the nephron and collecting system. The lesions have been related specifically to various segments,[1, 3-15] or described diffusely throughout all tubules.[2]

This divergence of opinion may be explained by several factors. There was considerable variation in the degree and duration of hypercalcemia; in most instances it was of long duration so that renal lesions were chronic and early primary changes were not apparent. Moreover, alterations in the composition of extracellular fluid and the acid-base balance in prolonged hypercalcemia due to various causes might well have affected the morphologic response in the kidney. As to the localization of lesions in the nephron or collecting system, it is impossible in conventional histologic sections to identify tubular segments which have undergone pathologic alterations, and, furthermore, the continuity of a given lesion cannot be traced throughout its entire course. In such situations, the location and extent of renal lesions can be determined only by the microdissection technique.

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Data to be presented in this report indicate that in dogs, hypercalcemia of brief duration induced by parathyroid extract causes renal lesions of specific nature and localization. These are associated with selective impairment of concentrating ability, and the structural alterations may lead to chronic renal damage and functional insufficiency.

Methods

Nine mature female dogs (Table I), weighing 8 to 22 kg., were maintained on Purina Chow supplemented with horse meat. Prior to an experiment, food and water were withheld for 24 hours, and on the evening before an experiment, 5 units of vasopressin in oil were administered subcutaneously. Sixty to 97 units of parathyroid extract per kg. of body weight were injected subcutaneously in 3 divided doses during the 24 hours preceding the study. A control study was carried out on each dog one week before it received parathormone; during this time vasopressin alone was administered. Functional investigations were done during mannitol diuresis with the addition of vasopressin; the complete details have been described elsewhere.[16] Several animals had repeated functional studies for periods up to 44 days after parathormone treatment. Inulin was determined by the method of Walser, Davidson and Orloff,[17] osmolarity with a Fiske osmometer, serum calcium according to Kingsley and Robnett,[18] and urea by the method of Conway.[19]

Necropsy was performed on the dogs at intervals up to 44 days after receiving parathormone. Kidneys for tissue analysis were quick-frozen in alcohol-dry ice, and samples of cortex and medulla were obtained by sharp dissection in a cold room at 4°C. Kidneys were fixed in formalin and alcohol, and histologic staining in addition to hematoxylin and eosin included the Masson trichrome stain, the periodic acid-Schiff (PAS) stain, the von Kossa procedure and various alizarin dyes for staining of calcium; fat was stained by flaming red [20] and Sudan IV.

The technique of microdissection was that of Oliver and co-workers; this has been described previously.[21] All dissected tubular segments were examined by phase contrast microscopy, and in many instances they were stained with iron hematoxylin, flaming red or Sudan IV. Notes and drawings were made on dissected specimens, and typical structural changes were photographed. Continuous comparisons were made between the characteristics of lesions in histologic sections and microdissected tissue. For purposes of illustration, tubular segments were constructed from overlapping serial photomicrographs taken of unstained specimens by phase contrast microscopy. Since phase contrast micrography produced a bright halo effect, outlining the tubular segments, and a dark background, the latter was cut away with a razor blade prior to mounting of the prints.

TABLE I

EFFECTS OF PARATHYROID EXTRACT ON RENAL FUNCTION AND STRUCTURE

Dog no.	Procedure	Serum Ca mg./100 ml.	T^cH_2O ml./min.	Max. $\frac{Uosm}{Posm}$	C_{In} ml./min.	BUN mg./100 ml.	Necropsy days after PTE	Degree of structural alterations: Obstruction	Hyaline casts	Fatty change	Atrophy, prox. convol.
5	Control	10.5 *	4.5		80	11.2					
	97 units PTE/kg.	15.0	−0.4		13	35.6	1	3+	3+	0	0
8	Control	10.4	3.6	4.9	70	10.9					
	60 units PTE/kg.	16.0	−0.4	1.4	34	21.2	1	1+	1+	0	0
44	Control	10.6	2.7	7.2	43.8						
	90 units PTE/kg.	18.6	−0.3	1.2	12.5		1	2+	2+	0	0
46	Control	10.0	3.5	6.2	39.8						
	90 units PTE/kg.	18.8	0.3	1.2	6.5		1	2+	2+	0	0
43	Control	10.2	4.5	6.5	55.1						
	90 units PTE/kg.	16.4	−0.5	1.5	33.3		5	2+	0	3+	1+
6	Control	8.7	3.2		49	14.8					
	94 units PTE/kg.	13.0	−0.5		22	26.0					
	9 days later	9.2				37.0	9	3+	0	3+	1+
19	Control	9.4	3.9	3.0	49	22.5					
	60 units PTE/kg.	14.5	0.2	1.1	10	43.9					
	8 days later	7.0	0.1	1.8	13	78.4					
	23 days later	7.6	0.4	2.5	26	31.4	23	3+	0	2+	2+
4	Control	8.7	4.3		65	15.1					
	77 units PTE/kg.	14.7	2.7		83	19.0					
	31 days later	10.1				26.6	31	1+	0	1+	1+
3	Control	10.3	0.5		29	12.0					
	90 units PTE/kg.	16.5	−1.1		27	13.3					
	9 days later	10.0	1.0		28	24.3					
	44 days later	11.7				27.4	44	1+	0	0	0

Key: T^cH_2O = Tubular reabsorption of water free of solute. A negative value indicates urine hypotonic to plasma.

Max. $\frac{Uosm}{Posm}$ = Maximum urinary concentration ratio

C_{In} = Inulin clearance.

BUN = Blood urea nitrogen.

PTE = Parathyroid extract.

* Figures for serum calcium denote serum calcium before treatment (control), after treatment with parathyroid extract, and at varying times following recovery or correction of hypercalcemia.

Results

Functional Studies

These investigations have been fully described elsewhere,[16] and only data useful for a pathophysiologic correlation will be discussed.

The subcutaneous injection of 60 to 97 units of parathyroid extract per kg. of body weight over a 24-hour period resulted in a marked increase in serum calcium in all animals and significant reductions in tubular reabsorption of solute-free water (T^cH_2O) and the maximum urinary concentration ratio. These observations indicated diminished ability of the tubules to reabsorb water (Table I). In 6 of 9 animals, the urine became more dilute than plasma. The glomerular filtration rate was usually decreased, but remained unchanged in one animal and rose in another. Serum calcium levels gradually fell to normal in 48 to 72 hours, and were associated with a return of T^cH_2O to essentially control levels except in dog 19. This animal also showed a persistently low glomerular filtration rate and an elevated blood urea nitrogen. Blood urea nitrogen was elevated and tended to rise further even after injections of parathyroid extract were discontinued.

Lower doses of parathyroid extract (30 to 35 units per kg.) in 2 dogs produced only a slight elevation of serum calcium, with no change in concentrating ability or glomerular filtration rate. In 3 animals, the subcutaneous injection of 4.5 to 7.0 gm. of calcium as calcium acetate caused slight elevation of serum calcium for up to 4 hours, but this had returned to normal by 24 hours when T^cH_2O was unaltered.

Morphologic Features

Structural alterations in the kidneys were examined by conventional histologic and microdissection methods and were classified as acute or chronic.

Acute Lesions. These were present 24 hours after parathormone and had a specific distribution. They were seen in the distal nephron beyond the thin limb of Henle (Fig. 1), i.e., in the ascending limb of Henle, the distal convolution with its connecting limb, and throughout the entire collecting system (Fig. 2). In the latter they were more numerous and of greater severity. The lesions occurred as small foci, usually involving only short tubular segments, and were of patchy distribution. They consisted of tubular epithelial degenerative changes leading to necrosis, often with calcification of the epithelial cells and the adjacent basement membranes. The necrotic and calcified cellular debris frequently formed obstructing intratubular casts, leading to dilatation of the tubules proximally.

Degenerative change appeared as hydropic swelling of the epithelial cells (Figs. 2 and 3) which were 2 to 3 times enlarged and markedly distorted. Swelling was most prominent in the basal part of the cell, which was distended with fluid, and the remaining cellular organelles were displaced toward the lumen of the tubule. In microdissected material, nuclei which normally appear as clear circular spaces because of their low chromatin content were completely obscured. Vacuolation and droplet formation were not part of the degenerative process. Degenerative changes occurred either in isolated foci or were associated with necrotic lesions, with transitions from one to the other.

As with the degenerative alterations, necrosis involved only focal segments of the tubules (Figs. 1 to 5). Necrosis was characterized by complete disruption of tubular epithelium. The necrotic cellular debris was coarsely granular and contained large irregular calcific masses (Figs. 1 and 2). In some lesions entire desquamated cells were calcified, while in others the debris of disintegrated cells appeared as small uniform bodies, somewhat larger than mitochondria. The bodies stained for calcium and could, therefore, represent swollen and calcified cellular organelles. Basement membranes in regions of epithelial necrosis were frequently calcified (Fig. 5). However, in lesions at this stage, calcification did not affect the intertubular interstitium. In some animals there was slight interstitial exudation, with a few neutrophils accompanying the necrotic tubular lesions.

A common feature was displacement of necrotic cellular debris down the tubule, leaving the basement membranes completely devoid of epithelium (Fig. 5). With collapse, these might well be misinterpreted in conventional sections as thin limbs of Henle; however, their true nature was revealed by microdissection. They were most common in the medullary collecting tubules and appeared as segments comprised only of intact basement membranes. Although basement membranes were occasionally ruptured during microdissection, disruptions secondary to necrosis were not encountered in any of the acute lesions.

Two types of casts were encountered in kidneys with acute lesions. Those in regions of focal tubular necrosis were composed of calcified epithelial debris and were PAS-positive (Figs. 3 to 5). Those of the second type were uniformly homogeneous and eosinophilic, resembling hyaline casts. They were also PAS-positive, never calcified, and most often located in Henle's loop, proximal to foci of necrosis (Fig. 1). Tubules containing hyaline casts were not remarkable, save for slight dilatation and flattening of the epithelium (Fig. 1).

Dilatation occurred proximal to points of obstruction by casts (Figs. 1 and 3) and extended for variable distances up the collecting tubules,

along the distal convolutions and down the ascending limbs of Henle. Some segments were dilated to 3 to 4 times their normal diameter, with flattening of the epithelium and wide separation of the cells; the epithelial pattern, however, was normal in every respect, and cellular structure was unaltered.

Examination of glomeruli in animals with acute lesions revealed no structural changes. Proximal convolutions showed a uniform epithelial pattern with normal cellular detail. Terminal segments of proximal convolutions revealed slight cellular swelling without alteration of intracellular structures, possibly caused by osmotic diuresis occurring prior to death.

Chronic Lesions. These were seen in animals sacrificed between 5 and 44 days following administration of parathyroid extract. There were fatty degenerative change, proliferation in foci of necrosis, and persistence of many obstructive lesions.

Fatty alteration was seen throughout the ascending limb, distal convolutions and entire collecting system (Figs. 6 and 7), a pattern of distribution similar to that of the acute lesions. Lipid accumulation in these sites is abnormal since, in the dog, lipid occurs normally only in the distal third of the proximal convolution. The lipid was in droplets of variable size and was highly refractile; it took up such stains as Sudan IV and flaming red avidly. Most cells contained a few lipid droplets; however, in some segments, they were so numerous that the normal cellular structures were entirely obscured (Figs. 6 and 7).

The initial response in regions of tubular necrosis was proliferation of bizarre regenerating cells. These contained large vesicular nuclei, frequently two distinct nucleoli, and exhibited a scanty amount of cytoplasm (Fig. 8). With time, the nuclei became smaller and darker staining and the cytoplasm more abundant and flattened. Proliferative lesions in the connecting segments between distal convolutions and collecting tubules showed an increase of intercalated cells, which were detected by their small dark nuclei and abundant cytoplasm of irregular outline. Following epithelial necrosis in the ascending limbs of Henle, distal convolutions, and cortical collecting tubules, most affected segments became replaced by regenerating epithelium. As the cast material disappeared, the functional capabilities of the tubules became re-established. On the other hand, obstructive casts tended to persist in the medullary collecting system, where the intraluminal casts appeared firmly fixed to thickened basement membranes (Figs. 8 to 10). Regenerating epithelium grew into or, more often, completely around these casts (Figs. 8 and 12). In many regenerating foci in the collecting system, the proliferative response of the tubular epithelium was so marked that it often

resulted in tubular obstruction (Fig. 11). Tubules distal to points of obstruction showed collapse and atrophy (Fig. 9), and foci of epithelial necrosis and calcification in these segments often appeared interstitial in location.

Some chronic lesions in the medullary collecting tubules were not limited to the tubules but extended to the adjacent connective tissue in the form of calcification with associated proliferation of fibroblastic tissue (Figs. 6 and 12). This was so firmly attached to the collecting tubules that it could not be teased free during microdissection (Fig. 6). Segments of descending terminal proximal convolutions and ascending limbs of Henle were often caught in this fibrous tissue and could be dissected free only with great difficulty so that breakage was frequent. However, examination of many of these segments in partially or completely dissected specimens did not reveal any structural alterations.

Obstruction by casts and proliferated epithelium in the medullary collecting tubule led to dilatation proximally, which extended up the collecting segment and for variable distances along the distal part of the nephron (Fig. 7). In kidneys with many obstructive lesions, with time, atrophic alterations appeared in the proximal convolutions (Fig. 13). These were manifest by uniform decrease in caliber of the tubules, flattening of the epithelium and simplification of the convolutions. Glomeruli in animals with chronic lesions were normal. Vascular involvement was absent except in one kidney (dog 6) which showed focal calcification of peritubular capillaries in the medulla, associated with lesions in adjacent tubules.

Discussion

Hypercalcemia of brief duration produced by parathyroid extract caused renal tubular lesions of specific nature and distribution, with impairment of renal concentrating ability. The degree and duration of hypercalcemia must be important in the development of renal functional and structural alterations since elevated serum calcium of low magnitude, induced by parathyroid extract or the intravenous administration of calcium for less than 4 hours, did not produce renal lesions or markedly alter concentrating ability.[22]

Recent morphologic and physiologic studies have indicated that urine is concentrated in the collecting tubules.[23-25] In the present experiments, morphologic alterations occurred in the distal part of the nephron and the collecting tubules, and the lesions were of focal patchy distribution without uniform involvement of the collecting system. In some instances, detectable structural changes did not appear severe or widespread enough to account for the functional alterations which were

observed. This was also true of rats with hypercalcemia due to vitamin D intoxication.[26] In dog 3, renal concentrating ability returned to normal when serum calcium fell to physiologic levels, even though morphologic lesions persisted. Similar observations have been made in humans.[27-29] In another animal (dog 19) where T^cH_2O remained low after return of

TABLE II

CALCIUM CONTENT OF SERUM, URINE AND RENAL TISSUE OF DOGS TREATED WITH PARATHYROID EXTRACT, AND NORMAL DOGS

	Dog no.*	Serum Ca (mg. per 100 cc.)	Urine Ca (mg. per 100 cc.)	Ca content, mg./kg. FFWT †	
				Cortex	Medulla
Parathormone	8	16	6	179	832
	19	7	7	173	1007
Normal	24	6	2	188	141
	25	7	4	116	103

* Animals were sacrificed while undergoing mannitol diuresis.
† Fat-free wet tissue.

the serum calcium to a normal range, extensive renal structural lesions appeared to account for this. In some humans, as well, renal concentrating ability has not been observed to improve with correction of hypercalcemia.[29] The conclusion may be drawn that changes in renal structure may sometimes be of sufficient extent to explain diminished ability of the renal tubules to reabsorb water. However, this is not always the case, especially in early experimental lesions. In the latter instance, functional changes without obvious morphologic alterations are presumably induced by hypercalcemia.

The ionized fraction of serum calcium is elevated in hyperparathyroidism and vitamin D poisoning,[30] so that the quantity of calcium delivered to the renal tubules via the glomerular filtrate should be increased. Normally, if over half of the serum calcium is ionized,[30] more than 95 per cent of the filtered calcium must be reabsorbed, allowing 100 to 250 mg. for the daily urinary loss. Reabsorption in the proximal nephron probably accounts for much of this.[31] If the reabsorptive mechanisms in the proximal portions of the nephrons become saturated, excess calcium would pass on to more distal portions of the nephrons and collecting tubules. When this occurs, calcium appears to accumulate in epithelial cells and basement membranes. Tissue analysis (Table II) as well as morphologic investigation revealed a marked increase of calcium in the medulla following parathormone treatment.

Current concepts [23-25] of the mechanism by which urine is concentrated propose (a) active reabsorption of sodium by medullary tubules; (b)

increasing concentration of sodium (and urea) in medullary interstitial fluid along a rising gradient as the papillary tip is approached; and (c) permeability of the collecting ducts to water in the presence of vasopressin. An increased concentration of calcium in tubular fluid, interstitial fluid or intracellular water of the medulla might interfere with the renal concentrating mechanisms in a number of ways.

Calcium appears to alter cellular permeability through an effect upon the cell membrane. Many cells become more permeable in the absence of calcium, while increased calcium has an opposite effect.[32] The effects of calcium on nerve excitability and the heart beat are well known and have been ascribed to altered permeability of cell membranes, since they are rapidly reversible.[32] With hypercalcemia, decreased permeability of cell membranes or basement membranes preventing passive diffusion of water out of the collecting ducts may partially explain the defect in water reabsorption.

Excessive concentrations of calcium might have affected the metabolism of the tubular epithelium so as to impair the active transport of sodium into the interstitium of the medulla. Excess calcium in the liver cell inhibits oxidative phosphorylation,[33, 34] and it has been shown that calcium ions cause swelling of mitochondria under certain conditions.[35] Studies with the electron microscope have shown destruction of mitochondrial structure in renal tubular cells after large doses of parathyroid extract.[36] Such effects of calcium upon cell metabolism may account for the renal lesions which were noted, since impaired cellular metabolism can lead to hydropic change which is due in part to swollen mitochondrial elements.[37] With sustained injury, this is followed by cellular calcification and necrosis. Gallagher, Gupta, Judah and Rees[33] have reported such lesions in liver cells after thioacetamide administration and attributed them to the intracellular accumulation of calcium.

In our animals, impaired cellular metabolism could have existed in tubular segments after 24 hours of hypercalcemia without detectable structural alterations. In support of this view was the appearance, after 24 hours, of fatty change which had a distribution similar to the earlier degenerative and necrotic lesions.

The effect of potassium on cellular permeability and excitability is opposite to that of calcium.[32] Of interest is the fact that potassium depletion [38, 39] and hypercalcemia have closely related effects upon the kidney. Both produce a selective defect in the tubular reabsorption of water with structural alterations located in the medullary collecting tubules. In potassium depletion, however, the tubular lesions are more extensive and uniform in distribution.

Hypercalcemia caused a drop in glomerular filtration rate and a rise in

blood urea nitrogen in some animals (Table I). These functional changes were clearly related to the degree of intratubular obstruction. When serum calcium returned to normal levels, there was usually only a gradual improvement of glomerular filtration rate and blood urea nitrogen because of a persistence of obstructive lesions.

The obstructive casts were of two types, calcific and hyaline. Both were present in kidneys with acute lesions and were PAS-positive. The work of Engel [40] suggests that they contain mucopolysaccharide since he showed that parathormone releases mucoprotein residues of bone matrix ground substance into the blood, whence they reach the kidney. With cessation of parathormone, the hyaline casts disappeared, but calcific casts tended to remain fixed within the medullary collecting ducts.

It is possible for urine flow to be re-established around points of obstruction [41] in the collecting system by means of retrograde flow up a side duct above the block and then down a neighboring tubule so that the occluded segment is circumvented. This was unlikely in kidneys with extensive involvement since microdissection revealed that obstructive lesions were so numerous in some collecting duct arborizations that flow appeared blocked in all directions, thus accounting for renal insufficiency and atrophy of proximal convolutions.

Our observations indicate that hypercalcemia of brief duration can cause permanent renal damage with renal insufficiency and in some instances persistently impaired concentrating ability. Therefore, in clinical practice, hypercalcemia should be corrected quickly, and investigative studies or diagnostic tests requiring abnormal elevation of serum calcium should be discouraged.

Interstitial calcification, when present in the kidney, was minimal. It should be stressed that the distribution of calcified cellular debris and basement membranes in obstructed tubules with atrophy could be misinterpreted as having interstitial location. Bell,[15] in a case of hyperparathyroidism, found that all the calcium was in the medulla and in the shape and direction of collecting tubules, thus concluding that the calcium was intratubular, not interstitial. Calcified masses in large ducts near the papillary tip would predispose to the formation of calculi.

Although, with prolonged hypercalcemia, calcification has been observed in proximal convolutions, glomeruli and blood vessels,[5, 7, 8, 26] the evidence presented here suggests that the earliest morphologic alterations occur in distal portions of the nephron and in the collecting tubules and are associated with severe impairment of concentrating ability. These findings are in accord with other observations imputing a major role in the concentrating process to the medullary tubules.

Summary

Hypercalcemia of brief duration induced in dogs by the administration of parathyroid extract for only one day produced structural alterations in the kidneys which were localized by microdissection to the ascending limb of Henle, the distal convolution, and the collecting system. These lesions were of patchy distribution and consisted of epithelial degeneration, calcification and necrosis, often resulting in intratubular obstructing casts. Lesions in the ascending limbs, distal convolutions and cortical collecting tubules usually were healed by regenerating epithelium. Those in the medullary collecting tubules tended to remain as fixed intratubular calcific casts which produced marked dilatation proximally and, in some instances, atrophy of proximal convolutions. Calcification was principally intratubular, not interstitial.

Hypercalcemia of 24 hours' duration produced severe impairment of renal concentrating ability. In most animals there was a drop in glomerular filtration rate and a rise in blood urea nitrogen which was related to the degree of intratubular obstruction. It is postulated that the defect in tubular reabsorption of water is due to the inhibitory effect of excess calcium upon cellular permeability and metabolism. Kidneys with marked structural alterations showed persistent evidence of functional insufficiency. It is stressed that in clinical practice every effort should be made to correct and avoid hypercalcemia and maintain the serum calcium within the normal range.

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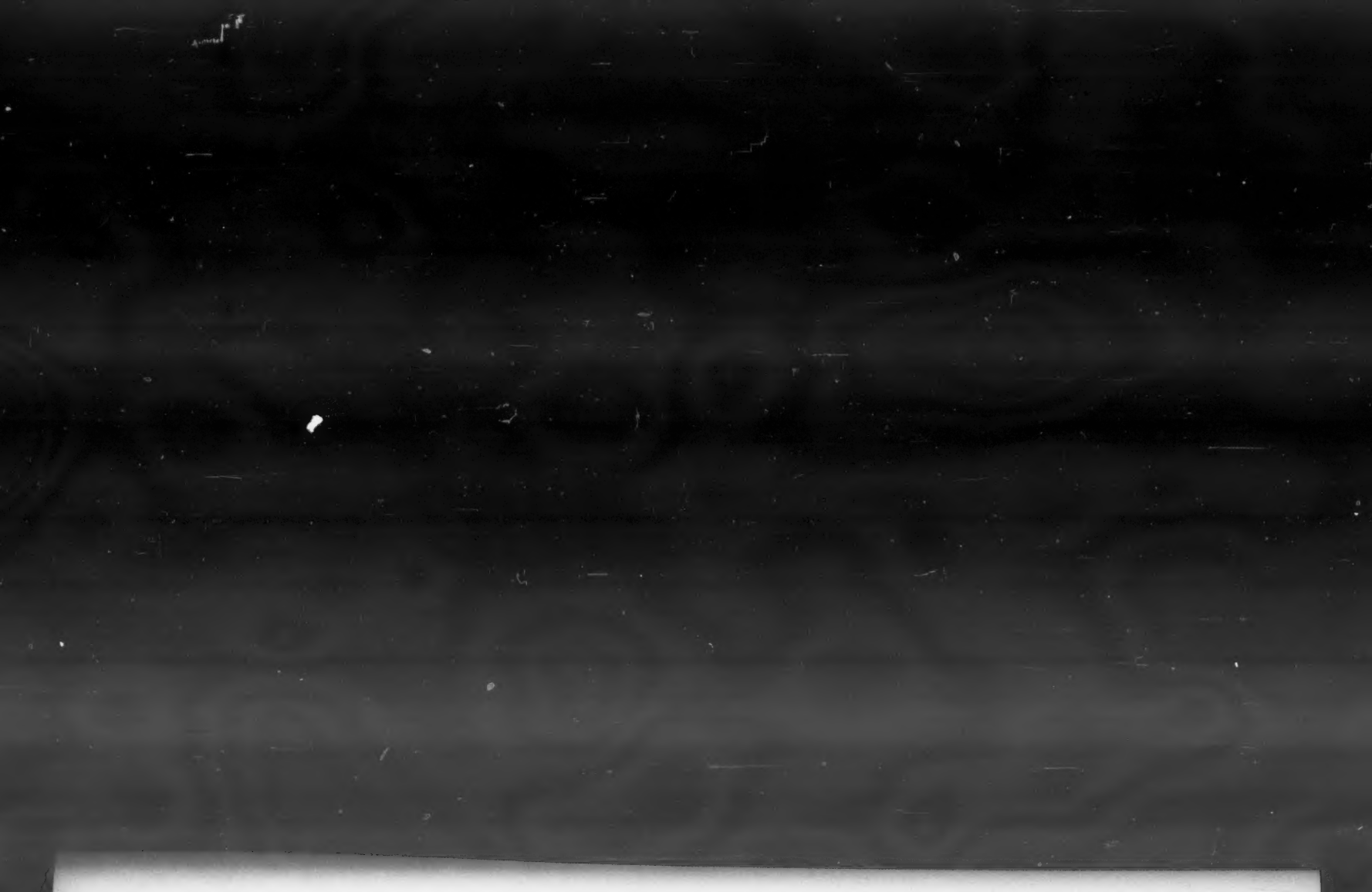
Sincere thanks are due to Dr. Jean Oliver and Sir Roy Cameron for their generous assistance.

[*Illustrations follow*]

Legends for Figures

FIG. 1. Necrotic lesions in ascending limbs of Henle. "A" is a loop of Henle. Between "L" and "L_1" the epithelium is necrotic and consists only of granular cellular debris which fills the lumen. The basement membrane is intact. Further up, the tubular epithelium gradually assumes a normal appearance and is comprised of a single layer of cells of uniform pattern. "B" and "C" are parts of the same ascending limb. The segment within the bracket shows epithelial necrosis and calcification with tubular obstruction. Proximally (segment "C"), the tubule is dilated and the lumen filled by a hyaline cast. The basement membrane is unaffected, and the epithelium in the dilated segment is normal except for minimal flattening. Dog 5, one day after parathyroid extract (PTE). Segment A, × 50; segments B and C, × 100.



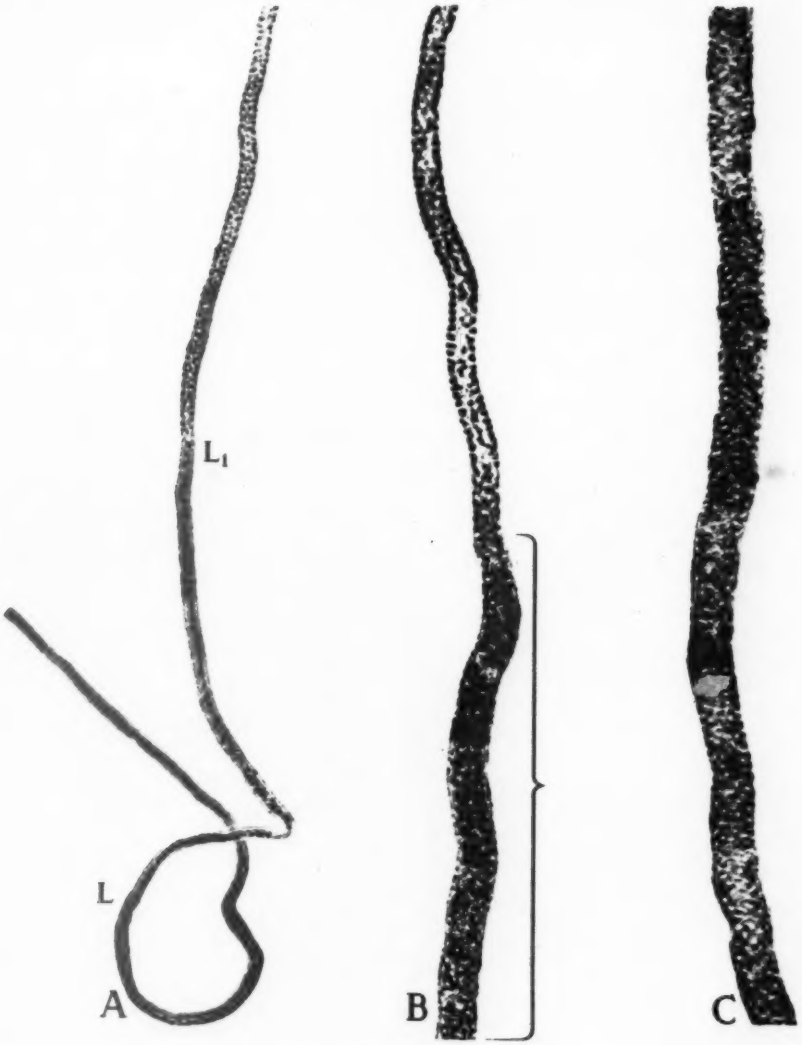


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FIG. 2. Necrotic lesions in terminal segments of distal convolutions and collecting tubules. "A" and "B" are cortical collecting tubules. Small foci of necrosis are indicated by the arrows. A larger segment of necrosis with abundant calcification is outlined by the bracket. At "D" the cells show hydropic swelling. "C" is a medullary collecting tubule with a focus of epithelial necrosis and calcification. Dog 5, one day after PTE. × 100.

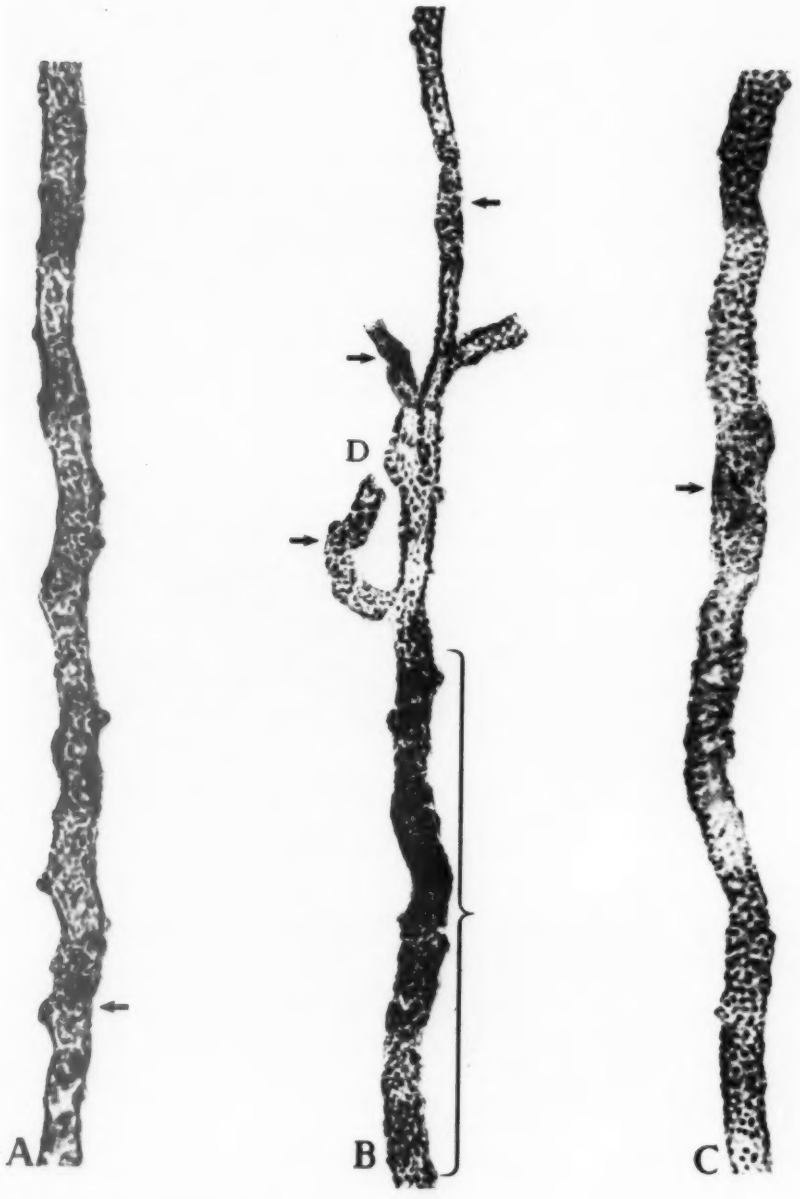

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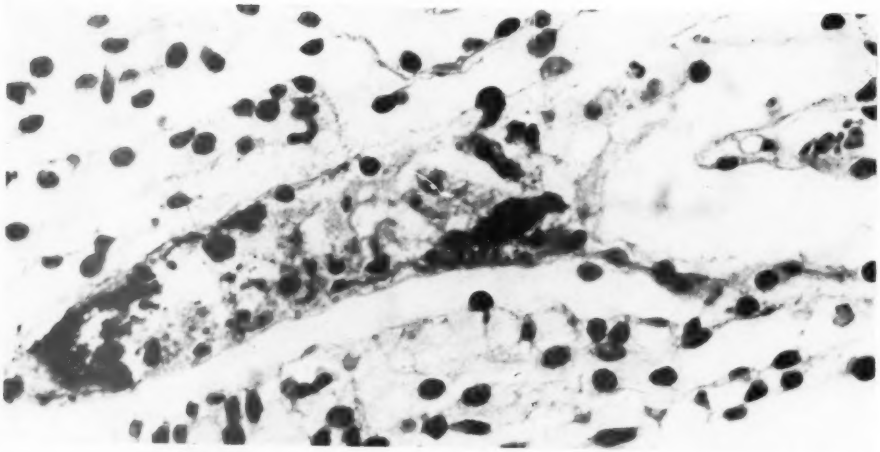
FIG. 3. A focus of necrosis in a medullary collecting tubule. The epithelial cells show swelling, calcification and necrosis with the formation of an obstructing cast and slight proximal dilatation. There is no involvement of intertubular tissue. Dog 5, one day after PTE. Hematoxylin and eosin stain. × 615.

FIG. 4. A focus of necrosis in a collecting duct made up of granular, calcified cellular debris. Dog 44, one day after PTE. Hematoxylin and eosin stain. × 615.

FIG. 5. Epithelial calcification and necrosis in a collecting duct with prominent calcification of the basement membrane. Much of the tubule is denuded of epithelium. Dog 46, one day after PTE. Periodic acid-Schiff stain. × 615.



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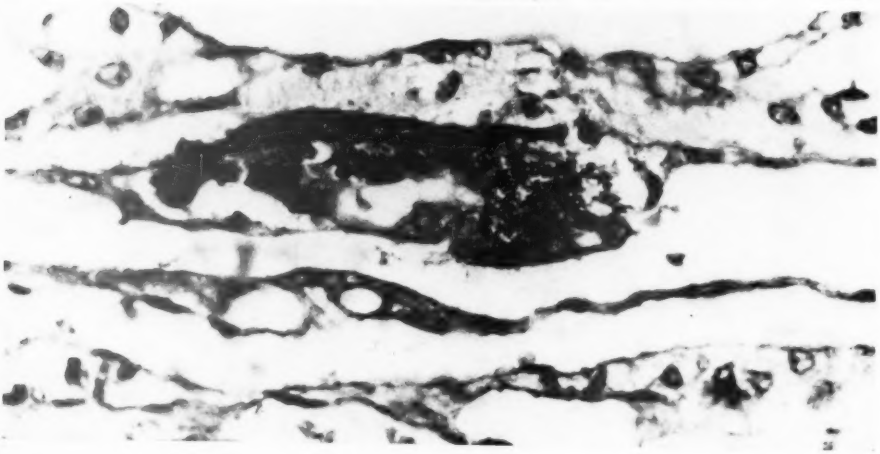

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FIG. 6. Obstructive lesions and fatty change in medullary collecting tubules. "L_1" and "L_3" represent fixed intratubular calcified casts and proliferation of interstitial connective tissue which is adherent to the tubules. In segment "B" there are two obstructing casts at points labeled "L_2." Fatty change consisting of small dark droplets of intracellular lipid can be seen in all segments but is more prominent in the regions indicated by the arrows. Dog 6, 9 days after PTE. × 100.

6

FIG. 7. Dilatation and fatty change of a cortical collecting tubule, distal convolution and ascending limb of Henle ("AL"). In places, the distal convolution is 5 to 6 times enlarged. Fatty change is of diffuse distribution but more marked in some segments (arrows). In the first part of the distal convolution, the epithelium is packed with lipid droplets so that the normal cellular detail is entirely obscured. Dog 19, 23 days after PTE. × 100.

ب

7

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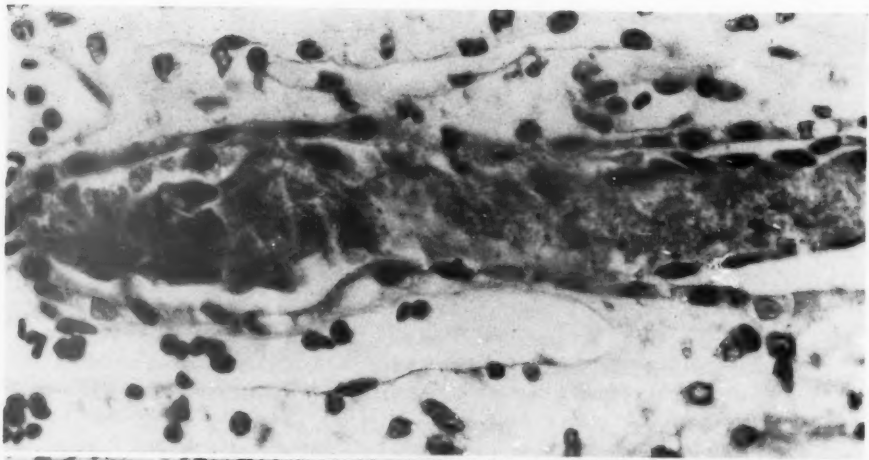
FIG. 8. A fixed calcified cast in a medullary collecting tubule producing obstruction. Flattened regenerating epithelium has grown entirely around the cast. There is no interstitial involvement. Dog 6, 9 days after PTE. Hematoxylin and eosin stain. × 615.

FIG. 9. Fixed calcific casts blocking two collecting ducts at their point of union. There are proximal dilatation and distal collapse. Note the absence of interstitial reaction. Dog 6, 9 days after PTE. Hematoxylin and eosin stain. × 140.

FIG. 10. A fixed cast in a medullary collecting tubule, causing obstruction and proximal dilatation. The cast consists of a cluster of proliferated, completely calcified cells. Dog 4, 31 days after PTE. Hematoxylin and eosin stain. × 615.



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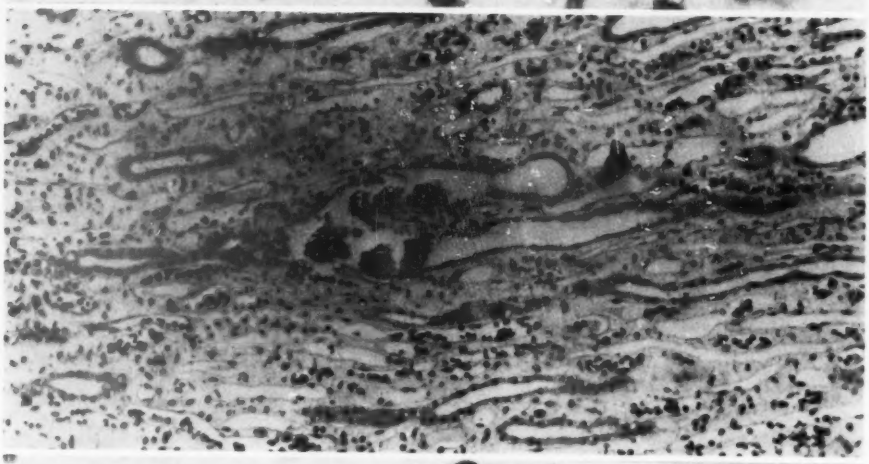

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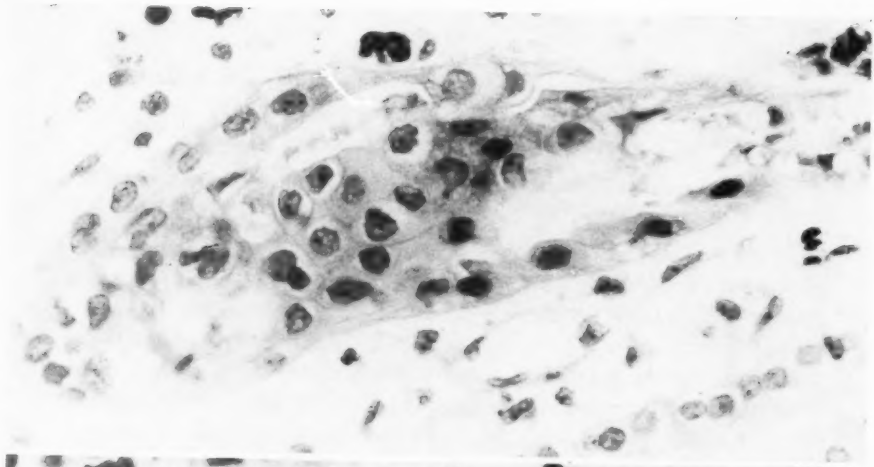
FIG. 11. Epithelial proliferation producing complete obstruction in a duct of Bellini. The proximal collecting duct was markedly dilated. Dog 3, 44 days after PTE. Hematoxylin and eosin stain. × 615.

FIG. 12. Two chronic lesions in a medullary collecting duct. On the left there is involvement of the interstitial tissue, with proliferation of connective tissue and an accumulation of mononuclear cells. Regenerating tubular epithelium has grown around this lesion with re-establishment of the tubular lumen. The lesion on the right is principally intratubular and forms an obstructing mass. Dog 19, 23 days after PTE. Hematoxylin and eosin stain. ×615.

FIG. 13. Marked dilatation is manifest in the collecting tubules and distal convolutions in the outer cortex. There is atrophy of proximal convolutions. Dog 19, 23 days after PTE. Hematoxylin and eosin stain. × 140.



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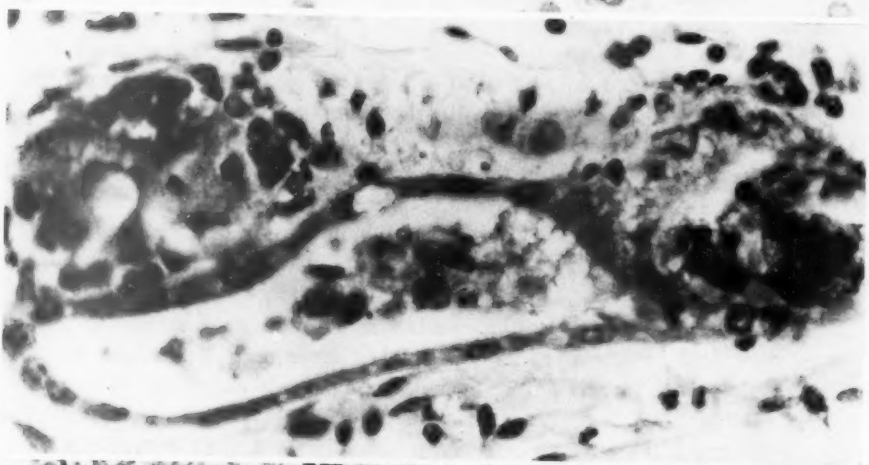

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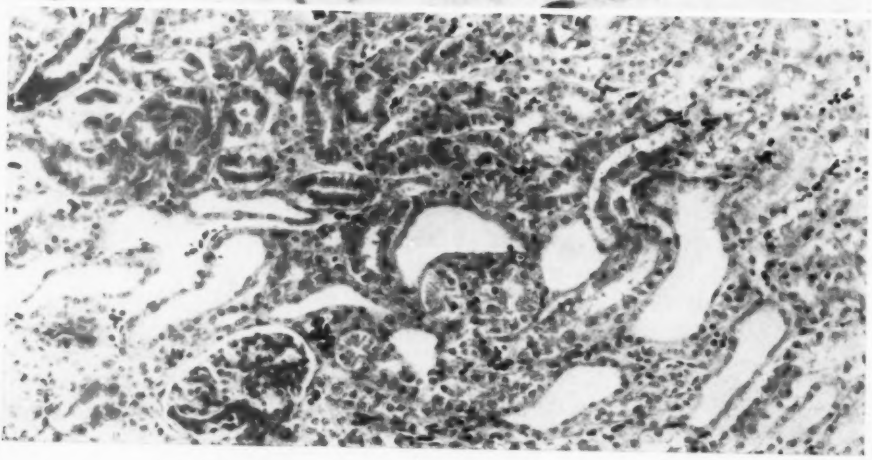

13

PRESENCE OF A TUMOR INHIBITORY PRINCIPLE IN LIVERS

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In investigating the effect of animal serums on the Gardner lymphosarcoma 6C3HED carried subcutaneously by C3H mice, we reported [1] complete and consistent inhibition of growth with guinea pig serum, slight and inconsistent inhibition with sheep and human serums, and no inhibition with rat, chicken, dog, bovine, calf, horse, or rabbit serums. Because of the slight but equivocal activity of sheep and human serums, we stated [2] that the tumor inhibitory principle (TIP) was not necessarily peculiar to guinea pig serum but might also be present in other animal serums, albeit in smaller amounts. If this was so, however, its demonstration had to await the development of a concentration procedure or the actual identification and isolation of TIP. In connection with the latter, our investigations [1, 3, 4] have indicated that TIP is present in the fluid and not the cellular portion of guinea pig blood, that it withstands heat at 66° C. for 30 minutes, that it is extractable with methanol, and that it exists in the globulin fraction of the serum. They have further indicated that the principle does not appear to be complement, properdin, or a lipopolysaccharide.

While studies concerned with the identification and isolation of TIP are continuing, it appeared to be of interest to determine, first, whether any of the organs of the guinea pig contained TIP and could thus serve as a source of this material in serum, and, second, if they did, whether similar organs of other animals might also contain TIP. Accordingly, in preliminary investigations two preparations were made of the following guinea pig organs: liver, spleen, kidney, lung, heart, and muscle. One preparation consisted of a suspension, in physiologic saline, of dessicated and ball-mill powdered tissues while the other consisted of a Waring blender slurry of fresh organs. Chloramphenicol and penicillin were added to each to insure bacteriostasis. The preparations were injected subcutaneously and intraperitoneally into C3H mice carrying a 4-day-old 6C3HED tumor. The observations indicated that the suspension of liver tissue alone exhibited tumor inhibitory properties and, conversely, that suspensions of spleen, kidney, lung, heart, and muscle showed none.

With these results as a background, extracts of guinea pig, sheep, rab-

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bit, bovine, horse, hog, and human livers were examined for tumor inhibitory properties. The following is a report of our findings.

Material and Methods

The tumor-host system consisted of the Gardner lymphosarcoma 6C3HED carried by C3H mice. The mice weighed about 20 gm. each and were purchased from the Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine. The donor tumor was 8 days old and was implanted into the subcutaneous tissue of the right flank by the usual trocar method. Treatment, either intravenously (i.v.) or intraperitoneally (i.p.) was begun on the fourth day after implantation, at which time the tumors measured 7 mm. in diameter. Intravenous injections of the extract of guinea pig liver were given on the fourth, sixth, and eighth days as single daily doses, while intravenous injections of extracts of other livers were given twice daily from the fourth to eighth days, inclusive, with one half of the dose administered in the morning and one half in the afternoon. All intraperitoneal injections were given as single daily doses.

The liver extracts were prepared under the supervision of Mrs. Leona Cappel, Cappel Laboratories, West Chester, Pennsylvania. Livers were rinsed 4 times in physiologic saline, and all extraneous tissue was removed. After adding physiologic saline in a volume equal to that of the liver substance, homogenization was achieved by means of the Waring blender and colloidal mill. The resulting mixture was centrifuged at 13,000 r.p.m. and filtered through Seitz filter pads of decreasing porosity (ending with pad S_3 with a porosity of 0.01 μ) to achieve clarity and sterility.

Because of the number of animals and injections involved, the experiments were conducted in groups. In each of these, controls consisted of an equal number of mice receiving no treatment, as well as an equal number treated at first with fresh guinea pig serum and later with extract of guinea pig liver. In the case of guinea pig materials, dosages used were near the lowest that would produce complete disappearance of tumor. In the case of other materials, dosages employed were the maximum tolerated. In either case, they were arrived at by trial and error in preliminary experiments.

Animals were weighed and tumors were measured on the fifth, sixth and ninth days after implantation, and all surviving animals were sacrificed 9 days after receiving the implants. Necropsy examinations were performed on all animals, and the tumor or tumor area, kidneys, liver, and lungs were examined at random or when they disclosed gross alterations. However, because it was impossible to distinguish between neoplastic lymphocytes and those of inflammatory nature, complete gross

TABLE I

EFFECT OF EXTRACTS OF SEVERAL MAMMALIAN LIVERS ON LYMPHOSARCOMA 6C3HED IN C3H MICE

Treatment	No. mice used	No. mice surviving 9 days	No. growths 9 days	Diameter of tumor (mm.) on days after implant.			Weight of mice (gm.)	
				4	6	9	Initial	9 days
None	48	48	48	7	13	19	20	24
1.0-1.4 ml. guinea pig i.v. every 2 days × 3	42	41	0	7	6	0	23	23
2 ml. sheep i.v. daily × 5	18	15	13	7	8	9	23	23
2.0-3.6 ml. hog i.v. daily × 5	32	21	20	7	7	11	20	20
2.4 ml. horse i.v. daily × 5	24	11	11	7	10	16	24	25
3.2 ml. rabbit i.v. daily × 5	12	12	12	7	9	11	24	26
1.2 ml. bovine i.v. daily × 5	12	10	10	7	7	16	22	22
2.0-2.8 ml. human #1 i.v. daily × 5	22	20	20	7	13	21	22	25
3.6 ml. human #2 i.v. daily × 5	12	12	12	7	11	20	20	25
1.0-1.4 ml. guinea pig i.p. daily × 5	30	29	0	7	6	0	21	19
3 ml. sheep i.p. daily × 5	6	6	3	7	6	7	21	22
2 ml. hog i.p. daily × 5	6	6	6	7	8	9	19	17
2.4-3.0 ml. horse i.p. daily × 5	10	5	5	7	9	15	21	23
3 ml. rabbit i.p. daily × 5	6	6	4	7	6	11	21	23
4 ml. bovine i.p. daily × 5	6	6	6	7	9	13	25	23
4 ml. human #1 i.p. daily × 5	6	5	5	7	10	16	21	22
3 ml. human #2 i.p. daily × 5	6	5	5	7	11	17	21	23

Key: i.v. = intravenously
i.p. = intraperitoneally
× 3 and × 5 = for 3 and for 5 doses

disappearance of the implanted growth rather than microscopic absence of lymphocytes was the criterion used for complete regression of tumor.

Results

The cumulated results of the experiments are outlined in Table I. As indicated, each of the 48 mice used as nontreated controls survived 9 days, and each developed a tumor that disclosed an average diameter of 13 mm. on the sixth day and 19 mm. on the ninth day. In the group treated intravenously, 41 of the 42 mice treated with extract of guinea pig liver survived 9 days; each showed complete regression of tumor. Of 18 mice treated with extract of sheep liver, 15 survived 9 days, 2 showing complete regression of tumor. The diameters of the remaining 13 tumors on the sixth and ninth days respectively, however, were only 8 mm. and 9 mm. Of 32 mice treated with extract of hog liver, 21 survived 9 days, and 1 showed complete regression of tumor. The diameters of the remaining 20 tumors on the sixth and ninth days respectively were 7 mm. and 11 mm. Of 24 mice treated with extract of horse liver, 11 survived 9 days, none showing complete regression of tumor. The diameters of the tumors on the sixth and ninth days respectively were 10 mm. and 16 mm. Of 12 mice treated with extract of rabbit liver, 12 survived 9 days, and none showed complete regression of tumor. The diameters of the tumors on the sixth and ninth days respectively, however, were only 9 mm. and 11 mm. Of 12 mice treated with extract of bovine liver, 10 survived 9 days, and none showed complete regression of tumor. The diameters of the tumors on the sixth and ninth days were 7 mm. and 16 mm. As noted, extracts of two human livers were tested. Extract #1 was from the liver of a white man, 50 years of age, who died of cerebral hemorrhage. Extract #2 was from the liver of a Negro woman, 34 years of age, who died from bronchial asthma. Of 22 mice treated with extract #1, 20 survived 9 days, and none showed complete regression of tumor. The diameters of the tumors on the sixth and ninth days were 13 mm. and 21 mm. Of 12 mice treated with extract #2, 12 survived 9 days, and none showed complete regression of tumor. The diameters of the tumors on the sixth and ninth days were 11 mm. and 20 mm.

In the group treated with intraperitoneal injections, of 30 mice receiving extract of guinea pig liver, 29 survived 9 days; each showed complete regression of tumor. Of 6 mice treated with extract of sheep liver, 6 survived 9 days, and 3 showed complete regression of tumor. The diameter of the remaining 3 tumors on the sixth and ninth days respectively were only 6 mm. and 7 mm. Of 6 mice treated with extract of hog liver, 6 survived 9 days, none showing complete regression of tumor. The diameters of the tumors on the sixth and ninth days were

only 8 mm. and 9 mm. Of 10 mice treated with extract of horse liver, 5 survived 9 days, and none showed complete regression of tumor. The diameters of the tumors on the sixth and ninth days were 9 mm. and 15 mm. Of 6 mice treated with extract of rabbit liver, 6 survived 9 days, and 2 showed complete regression of tumor. The diameters of the remaining 4 tumors on the sixth and ninth days were only 6 and 11 mm. Of 6 mice treated with extract of bovine liver, 6 survived 9 days, and none showed complete regression of tumor. The diameters of the tumors on the sixth and ninth days were 9 and 13 mm. Of 6 mice treated with extract of human liver #1, 5 survived 9 days, none showing complete regression of tumor. The diameters of the tumors on the sixth and ninth days were 10 mm. and 16 mm. Finally, of 6 mice treated with extract of human liver #2, 5 survived 9 days, and none showed complete regression of tumor. The diameters of the tumors on the sixth and ninth days were 11 mm. and 17 mm.

Histologic examinations of the tumors or tumor areas were not helpful. The growing tumors disclosed the usual masses of lymphoid cells while those that were regressing showed smaller numbers of these elements. Nuclear fragmentation was minimal, and massive necrosis was not encountered. Sections of the lungs of mice which died during intravenous injection showed extensive congestion and edema with scattered capillary "platelet" thrombi. Sections of the livers and kidneys disclosed only congestion. Animals receiving intraperitoneal injections showed some unabsorbed material, generally walled off by scanty deposits of fibrin. Leukocytic reaction to this was minimal.

With fresh guinea pig serum as a control, electrophoretic patterns were prepared on each of the liver extracts used in the experiments. All of the patterns were remarkably similar. They disclosed little or no albumin and an abundance of globulins. In decreasing order of frequency, the proportion of each of the globulins was roughly as follows: beta, alpha$_2$, alpha$_1$, and gamma. For the same quantity (16 gamma) of sample applied in each electrophoretic pattern, the globulin content of extract of guinea pig liver was considerably greater than that of other livers.

Discussion

From the data presented, it is obvious that the extract of guinea pig liver possesses excellent tumor inhibitory activity, since a dose as low as 1 ml. given intravenously or intraperitoneally induced complete regression of tumor in 72 out of 72 mice. Of the two routes of administration, the intravenous was more effective, for in most instances the tumor had, to all intents and purposes, disappeared after a single in-

jection, whereas 2 to 3 intraperitoneal injections were required to achieve the same effect. In data not tabulated in this report, it has further been determined that the extract of guinea pig liver (prepared as indicated) is as potent a tumor inhibitory agent as lyophilized guinea pig serum and only slightly less active than fresh guinea pig serum. Because of this, and because other guinea pig tissues showed no tumor inhibitory activity, it seems reasonable to assume that the source of TIP in guinea pig serum is the liver.

Although the results are less spectacular, there is no doubt that a tumor inhibitory principle (TIP) is also present in the liver of the sheep, hog, and rabbit, since treatment with extracts of each of these produced either marked retardation of growth or, in some instances, complete regression of the 6C3HED tumor. In an investigation of the serums previously reported,[2] serum from the sheep disclosed slight and inconsistent tumor inhibitory activity, thus indicating that the sheep may produce TIP. Serum from the rabbit, however, did not exhibit tumor inhibitory activity. In subsequent studies, it was found that serum from the hog, in maximum tolerated doses, likewise did not reveal tumor inhibitory activity, thus also tending to exclude the hog as a producer of TIP. Now, however, in view of the findings herein recorded, it can be stated with assurance that TIP is not peculiar to the guinea pig, but may be found in the sheep, hog, and rabbit.

While the data, as presented, indicated that extracts of horse and bovine livers contained little or no tumor inhibitory activity, there were occasional mice in each group that did disclose marked retardation of growth of tumor. Because, in experience with over 5,000 implantations, spontaneous retardation of growth in this tumor-host system did not occur, we feel quite confident that, could the dose be increased, distinct tumor inhibitory activity could also be demonstrated in extracts of the livers from these two species.

Finally, the data would appear to indicate that extracts of the two human livers disclosed no tumor inhibitory activity. This too, however, may be a matter of dosage, for as prepared, all of the extracts except the guinea pig liver were lethal beyond the amounts administered. In the future, this limitation may perhaps be overcome when a method of concentration or isolation of TIP is available. Another factor causing inactivity of some of the extracts may be the age of the host from which the liver was obtained. Both of these considerations, namely, method of concentration or isolation of TIP, and age factor, are now being investigated. Since TIP has been demonstrated in livers from such diverse animals as the guinea pig, hog, and rabbit, and equivocally in livers from such animals as the horse and cow, and since electrophoretic patterns

of each of the extracts were similar, there is little reason to think that TIP is absent from the human liver. This, however, remains to be proved. If it should happen to be so, the implications and possibilities are manifold.

Summary

Physiologic saline extracts of livers from several species of animals were tested for tumor inhibitory activity against Gardner lymphosarcoma 6C3HED carried subcutaneously by C3H mice. Complete regression of growth was obtained with extract of guinea pig liver; marked but incomplete retardation of growth was induced by extracts of sheep, hog, and rabbit livers; slight and inconsistent retardation was demonstrated with extracts of horse and bovine livers, and no retardation was shown with extracts of two human livers. Electrophoretic patterns of each of the extracts showed little or no albumin and an abundance of globulin.

It is thought that the discrepancy in results may be due to limitation of dosage and perhaps to age of the host supplying the liver. These factors are now being investigated.

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THE RESULTS OF VARYING DEGREES OF NARROWING IN THE LEFT CIRCUMFLEX CORONARY ARTERY IN DOGS

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This is a report of the results obtained when the left circumflex coronary artery of the dog was narrowed without producing complete occlusion of the lumen. For control purposes, the 3 major coronary arteries were occluded completely by means of silk sutures applied 4 to 10 mm. from the origin of each vessel. In the case of the anterior descending artery, the tie was always applied distal to the origin of the anterior septal artery. In the case of the left circumflex artery, the tie was applied distal to the first atrial wall branch and proximal to the first ventricular wall branch. The mortality results of these experiments are shown in Table I.

It was clearly of no value to use the right coronary artery for narrowing experiments, since in our hands no mortality occurred following complete occlusion. It was decided not to use the anterior descending branch of the left coronary artery for the experiments because mortality results were variable. Different workers have reported mortality figures varying from as little as 10 per cent to as much as 90 per cent.[1-9] In our experience the size of infarcts produced following anterior descending artery ligation varied considerably.

The left circumflex artery was, therefore, chosen for investigation. When ligation is performed at the site described, mortality is very high[10-14] (Table I), and death usually occurs within 24 hours of operation. Twelve of our 14 dogs died within this period. Each of the 2 dogs which survived showed a very large infarct involving the left lateral and posterior wall of the heart and also the posterior part of the interventricular septum. This portion of the septum receives its blood supply from the posterior septal artery which arises from the terminal part of the left circumflex artery at the point of origin of the posterior descending artery. In a previous series of experiments[10-12] it was shown that occlusion of this vessel led to localized infarction of the posterior portion of the interventricular septum with resulting damage to the atrioventricular node and bundle of His, and the production of varying degrees of heart block. Five of the 14 dogs in which the left circumflex artery was occluded developed first or second degree heart block. It seemed reasonable to suppose, therefore, that by making use of the left

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circumflex artery, it would be possible to assess the results of narrowing in terms of mortality, the size of the infarcts produced, the presence of infarction of the interventricular septum, and the development of rhythm abnormalities as judged by electrocardiograms.

TABLE I

MORTALITY FROM CORONARY ARTERY OCCLUSION

Operation; artery occluded	No. of cases	Death from operation
Right coronary	10	0 (0%)
Anterior descending	20	8 (40%)
Left circumflex	14	12 (85.7%)

MATERIAL AND METHODS

Unselected mongrel dogs of both sexes, weighing between 22 and 34 pounds, were used for these experiments. Basal anesthesia was maintained with intravenous 6.5 per cent sodium pentobarbital (1 ml. per 5 pounds of body weight). A thoracic incision was made through the fourth left intercostal space. The pericardium was opened, the left atrium retracted with a spring clip, and a 2-0 silk suture was passed around the proximal portion of the artery with an aneurysm needle. A blunted hypodermic needle, bent to an angle of 135°, was then laid alongside the vessel, and the ligature securely tied about both the needle and the vessel; the former was then removed (Fig. 1). In this way the lumen of the artery was narrowed to the diameter of the needle used. Care was taken to perform the last part of the procedure as quickly as possible so that the artery would remain completely occluded for a minimum period. It is estimated that blood flow along the vessel was stopped completely for only 5 to 10 seconds at the maximum. Eighteen, 20 and 22-gauge hypodermic needles were used (Table II).

In the controls (5 dogs), all of the steps of the operation were performed, including occlusion of the artery, for 5 to 10 seconds, but the ligature was not tied. In no case was lasting damage produced. Temporary ST segment depression was noted in electrocardiograms procured during the period of occlusion; there was return to normal within one hour after compression was released. No further abnormalities were noted, and when the animals were sacrificed after 7 days, no gross or microscopic evidence of any muscle damage could be found. In neither the experimental nor the control series were thrombi found in relation to the region of narrowing.

The dogs were sacrificed on the seventh day following the operation. Lead II electrocardiograms were recorded before, during, and at inter-

vals after the operation until the time for sacrifice. Animals which survived were exercised twice a day after the second postoperative day and fed once daily. No attempts were made to perform exercise tolerance tests. In each case, immediately after sacrifice, the left circumflex artery was dissected and opened carefully distally to the point of constriction. A stylus of the size used for the narrowing procedure was then passed through the zone of the ligation until it emerged in the aorta (Fig. 2). In this way the size of the lumen, its patency, and the absence of thrombi were demonstrated in all cases.

Results

Narrowing With the 22-Gauge Needle (Group A)

This operation was performed 11 times, and 7 animals (63.6 per cent) died. Four died within 20 minutes of ligation, and the other 3 died at 5, 10, and 18 hours. The dog dying after 18 hours showed early acute infarction of the lateral and posterior walls of the heart, including the

Table II

EFFECTS OF NARROWING LEFT CIRCUMFLEX CORONARY ARTERY

Size of needle used	No. of cases	Death from operation	Atrioventricular block on electrocardiogram
22-gauge	11	7 (63.6%)	45.5%
20-gauge	10	4 (40%)	33%
18-gauge	20	5 (25%)	10%

posterior part of the interventricular septum. The others revealed no detectable alterations. Two of the dogs had complete heart block before death. The others had striking ST segment depression with ventricular extrasystoles proceeding to ventricular fibrillation. When sacrificed on the seventh day, each of the 4 animals which survived had large infarcts involving the lateral and posterior walls of the heart and the posterior part of the interventricular septum. Microscopically, typical healing infarcts and evidence of damage to the atrioventricular node and bundle of His were seen. In each dog striking ST segment depression had persisted until sacrifice, and 3 showed first and second degree heart block between 24 to 48 hours; this subsequently disappeared.

Narrowing With the 20-Gauge Needle (Group B)

This operation was performed 10 times, and 4 animals died (40 per cent). One died within 20 minutes, 1 at 9 hours, and 2 at 18 hours. No gross evidence of infarction was seen in this group, but microscopically, the 2 which survived 18 hours revealed early acute myocardial infarction of the posterior wall and interventricular septum with neutrophils.

One of the dogs which survived 18 hours had second degree heart block before death occurred. The others exhibited marked ST segment depression with ventricular extrasystoles proceeding to ventricular fibrillation.

The 6 surviving animals showed gross evidence of infarction, but only 3 were of the same order of magnitude as those encountered in Group A (Fig. 3). In the other 3 only the lateral wall of the left ventricle was affected. In all 6 dogs, the microscopic pattern was that of typical healing infarction, and the 3 larger lesions showed patchy involvement of the atrioventricular node and the bundle of His. Two dogs had second degree block which disappeared after 48 hours, and one had runs of ventricular extrasystoles 4 to 10 hours postoperatively; these subsequently ceased. All the dogs exhibited ST segment depression which persisted but showed a tendency to diminish in degree by the time of sacrifice.

Narrowing With the 18-Gauge Needle (Group C)

Of the 20 animals on which this operation was performed, 5 (25 per cent) died. Two died within 28 minutes, 2 at 18 hours, and 1 after 3½ days. The dog surviving 3½ days showed gross and microscopic evidence of infarction localized to the lateral wall of the left ventricle. Microscopic evidence of neutrophil infiltration of the lateral wall was seen in the 2 dogs which died after 18 hours. The other 2 hearts contained no demonstrable lesions. One of the 3 dogs which died after operation had complete heart block, varying from 3-1 to 8-1 over a period of 15 minutes before fibrillation occurred 20 minutes after the narrowing procedure was completed. The others had ST segment depression with ventricular extrasystoles proceeding to ventricular fibrillation.

Nine of the 15 animals which survived the procedure exhibited no gross evidence of an infarct. Microscopically, patchy fibrosis, replacing areas in which the muscle had been necrotic, could be seen scattered through the lateral and, to a lesser extent, the posterior walls in 5; in 4 there were no microscopic abnormalities. No evidence of damage to the interventricular septum was detected. Two of the survivors had small infarcts in the lateral wall of the left ventricle in relation to the zone of vascular narrowing. The remaining 4 dogs had large infarcts of the lateral and posterior walls of the left ventricle, and in 3 of these there was also involvement of the posterior part of the interventricular septum. Microscopically, there were healing infarcts, and damage to the atrioventricular node and the bundle of His area was observed in the 3 instances in which gross septal infarction had been noted. All the dogs in this group had ST segment depression in the initial stages; this returned to

normal after 3 to 4 days so that when the 3 dogs were sacrificed at 7 days, the electrocardiograms were considered normal. No gross infarcts were noted in any of these 3 dogs. One surviving animal had runs of ventricular extrasystoles 2 to 5 hours after the operation, and one dog developed second degree heart block at 24 hours after the operation; this reverted to normal rhythm at 48 hours. In both of these dogs there were gross infarcts, and the animal with heart block exhibited both gross and microscopic evidence of damage involving the atrioventricular node and bundle of His.

Discussion

It is felt that in causing arterial lumen encroachment in this way, a change is produced in regional blood flow to the myocardium which bears a resemblance to the effects of human atherosclerosis. The diameter of the left circumflex artery immediately proximal to the site of ligation was measured before the narrowing procedure and at necropsy. By comparing this figure with the diameter of the needle used, a rough approximation of the percentage of lumen remaining patent was determined. The figures were 60 per cent with the use of the 18-gauge needle, 42 per cent with the 20-gauge needle, and 33 per cent with the 22-gauge needle.

In these experiments 3 effects resulting from the ischemia of complete and partial occlusion of the left circumflex artery were investigated. These were the mortality rate, the size of the infarcts induced, and the electrocardiographic changes. The difference in mortality between complete occlusion (85.7 per cent) and narrowing with an 18-gauge needle (25 per cent) was striking. The correlation between increasing damage and degree of narrowing was to be expected, but it was established that infarction could be induced without complete obstruction of a coronary artery. The individual differences of response to the lumen narrowing were reminiscent of the effects of human coronary artery disease. It seems reasonable to suppose that the ability of one animal to recover completely while another developed a large infarct or died, depended on the maintenance of nutrition in the affected area. Whether this was the result of variation in the availability of collateral vessels or some other feature should be the object of further investigation.

Summary

The left circumflex coronary artery was narrowed by ligature in 41 dogs. A hypodermic needle was placed against the side of the coronary vessel, and a ligature tied around both the artery and the needle; thereafter the needle was removed. In this way the lumen of the vessel was

reduced to the size of the diameter of the needle. Three sizes of hypodermic needle (18, 20, and 22-gauge) were used, producing 3 degrees of narrowing. It was estimated that approximately 60 per cent, 42 per cent, and 33 per cent of the lumen remained patent following the use of the 18, 20, and 22-gauge needles respectively. In comparison with complete occlusion, it was found that the smaller the lumen after operation, the greater were the mortality rate, the size of the infarcts, and the electrocardiographic disturbances. A direct correlation between the degree of narrowing of the coronary artery and pathologic alterations was found.

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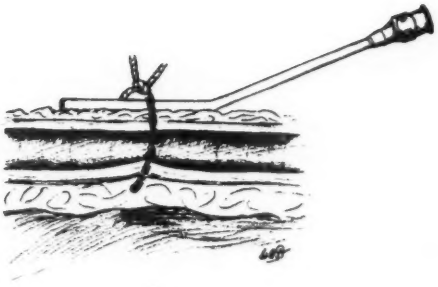
[*Illustrations follow*]

Legends for Figures

FIG. 1. Diagram of narrowing procedure. A. Suture around a vessel and a hypodermic needle. B. The suture is firmly tied. C. The needle is removed, leaving the vessel narrowed to the diameter of the hypodermic needle.



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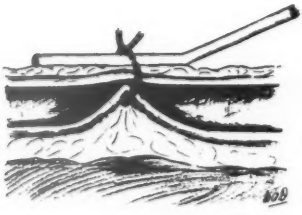

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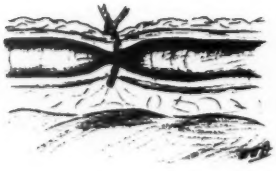

C

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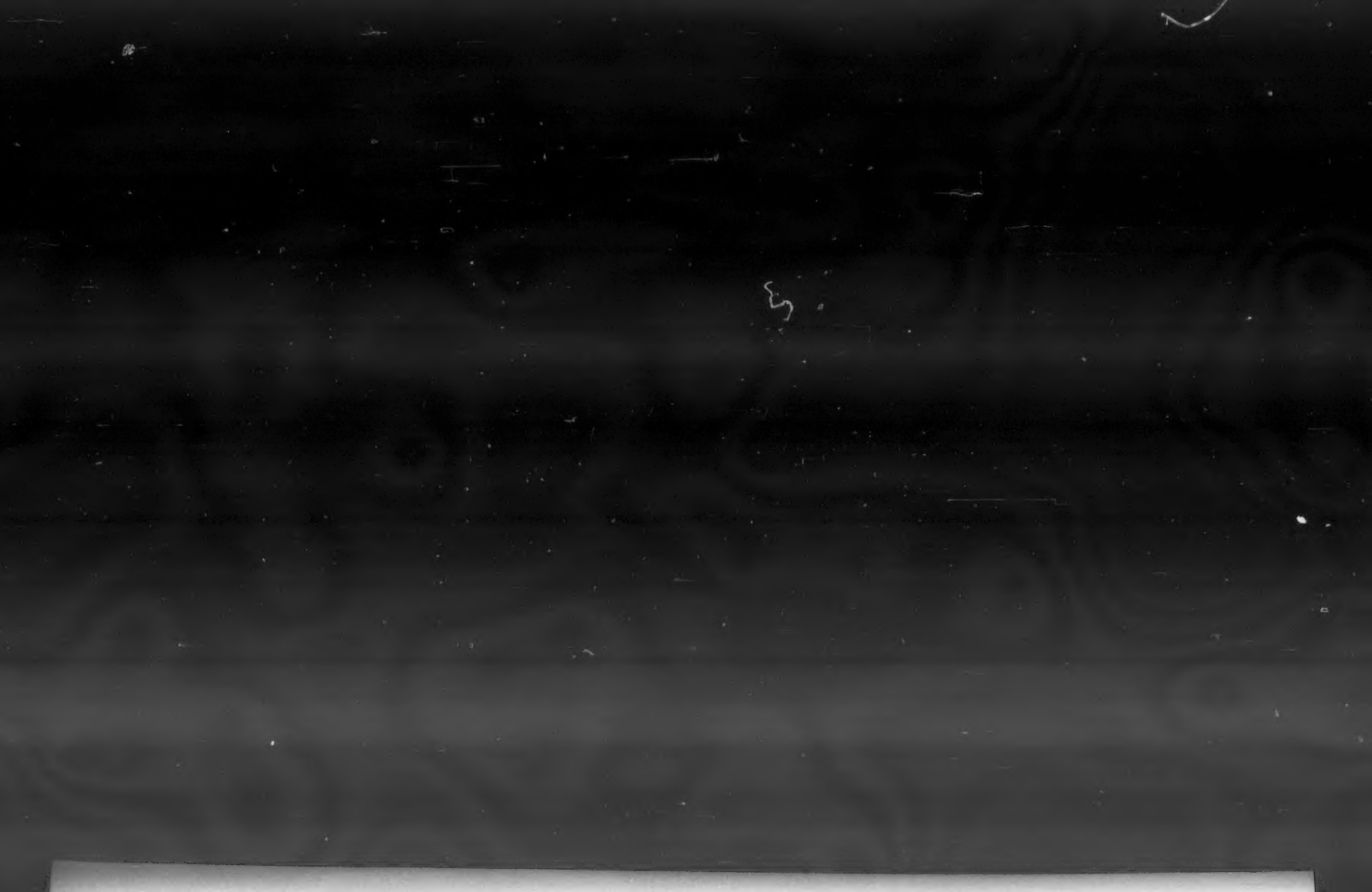
FIG. 2. A 20-gauge stylus passed through the area of narrowing in the left circumflex artery.

FIG. 3. Heart viewed posteriorly to show an infarct in the upper part of the interventricular septum following narrowing (20-gauge needle) of the left circumflex artery.



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